Temperature and infection by Metarhizium spp. in the desert locust

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Abstract

Metarhizium flavoviride is being developed as an entomopathogen for use against desert locust swarms. Field trials in Pakistan have revealed that temperature extremes may be a major limiting factor in the effectiveness of this mycoinsecticide (Bateman, pers. comm.). The aim of this work was therefore to investigate the interactions between temperature and the infection of the desert locust, *Schistocerca gregaria* (Forskal) by the entomopathogenic fungus, *M. flavoviride* isolate IMI 330189 and to study the possible roles of HSP70 in this process.

The effects of temperature on insect susceptibility to both fungal disease and chemical insecticides have been reported in a number of papers, but there have not been many reports of the effects of temperature when they are used in combination. Synergy between *M. flavoviride* and the benzophenyl urea insecticide, teflubenzuron, against third instar *S. gregaria* has previously been described in experiments carried out at the standard bioassay temperature of 28°C (Joshi, 1993). A number of bioassays were performed to extend this work to look at the effects of temperature on the mortality of third instar *S. gregaria* inoculated with *M. flavoviride*, teflubenzuron or both of these in a mixed formulation. It was found that the lowest LD_{50} and LT_{50} values for insects infected with *M. flavoviride* was obtained at 30°C. Temperatures above and below this gave rise to higher values. For applications of teflubenzuron it was found that the LD_{50} and LT_{50} values were for the most part inversely proportional to temperature. Combined applications of teflubenzuron and *M. flavoviride* did not show significant synergy except in bioassays carried out at 35°C. The reasons for this are discussed.

In order to further investigate the effects of temperature on *M. flavoviride*, growth, germination and appressorial formation studies were carried out both *in vitro* and *in vivo*. *In vitro* growth and germination were reduced by temperatures both above and below the optimum. For *in vivo* experiments conidia were stained with a fluorescent dye and applied to the locusts in aqueous formulations. In all cases optimal germination

was seen at 30°C, with a slightly lower percentage germination at 25°C and very little germination at 35°C. The ability to form appressoria *in vivo* also showed the same relationship with temperature. Investigations on the effects of cuticle on *M. flavoviride* revealed that conidia germinated fastest on the softer parts of cuticle, such as the hindwing, with germination on hard cuticle like that of the pronotum being much slower. It was also demonstrated that germination on dead locusts was faster than on live insects. Possible reasons for this difference are discussed.

Finally, the HSPs of *M. anisopliae* isolate ME1 and *M. flavoviride* isolate IMI 330189 were studied using molecular and biochemical techniques. Coomassie blue staining of SDS-PAGE gels of protein extracts from heat shocked *M. flavoviride* cultures revealed no consistent differences in banding patterns when compared to non-heat shocked controls. Western blots with a commercially obtained antibody to HSP70 however revealed an increase in the amount of the protein present after the a heat shock of 32°C. Interestingly, the quantity of HSP70-like polypeptides and number of isoforms detected was decreased after a heat shock at 45°C. Similar results were obtained for another two other isolates of *M. anisopliae*. ^{35}S methionine labeling of proteins produced during heat shock of *M. flavoviride* at 45°C revealed the strong induction of a protein of approximately 106kDa. At 32°C, a number of proteins were induced, but overall protein production changed little.

SDS-PAGE gels of haemolymph from infected adult male *S. gregaria* showed the induction and repression of a few proteins over the course of infection, mostly on the final day of infection. Much more substantial differences were detected when the haemolymph proteins produced on each day of infection were labeled with ^{35}S methionine. Western blots of infected *S. gregaria* haemolymph revealed the presence of very small quantities of HSP70 homologue circulating in the haemolymph on days 2 and 3 of infection.

PCR was used to amplify fragments of putative *hsp70* and *hsp90* genes using heterologous primers. The *M. anisopliae hsp70* PCR product was cloned and sequenced and shown to have substantial homology to other fungal *hsp70* genes. This gene appears to be a single copy which contains at least one intron. A similar gene has been found in *M. flavoviride* but has not yet been cloned. PCR has also been used to amplify regions of putative *hsp90*.

When RT-PCR was carried out on RNA samples extracted from *M. flavoviride* liquid cultures which had been subjected to a variety of different conditions, three different PCR products were seen corresponding to three mRNA species. Heat shock, starvation and cuticle in the media were each found to induce a different qualitative and quantitative response.

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Abbreviations

A_{260} ..... absorbance at 260nm
ATP ..... adenosine triphosphate
BCA ..... biological control agent
bp ..... nucleotide base pair
BPU ..... benzoylphenyl urea
Ci ..... Curies
DEPC ..... diethyl pyrocarbonate
DNA ..... deoxyribonucleic acid
HSP ..... heat shock protein
IPM ..... integrated pest management
kb ..... kilobase
kDa ..... kiloDalton
λ ..... lambda phage
LD_{50} ..... median dose taken to kill 50% of organisms in a given time
LT_{50} ..... median time taken to kill 50% of organisms for a given dose
M ..... Molar
MOPS ..... 3-(*N* morpholino)propanesulfonic acid
Mr ..... molecular mass
mRNA ..... messenger RNA
N ..... Normal
×g ..... gravitational pull
OD_{600} ..... optical density at 600nm wavelength
PAGE ..... polyacrylamide gel electrophoresis
PCR ..... polymerase chain reaction
pfu ..... plaque forming units
RNA ..... Ribonucleic acid
rpm ..... revolutions per minute
SDS ..... sodium dodecyl sulphate
Taq ..... *Thermus aquaticus* DNA polymerase
ULV ..... ultra low volume
UV ..... ultra violet

This thesis is dedicated to the memory of my great uncle Dr. Roland Heywood.

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Figures and Tables

1. Introduction

1.1 Locust plagues

Schistocerca gregaria is one of Africa's most devastating plague species, of all the locust species it affects the largest area and is the most polyphagous (Hill, 1979). Swarms of locusts can cover hundreds of square kilometres, consist of up to 50 billion insects and consume 70,000 tons of food daily, an amount equivalent to that consumed by the populations of New York, London, Los Angeles and Paris (Preston-Mafham, 1990). During the last swarm (1986-1987), locusts caused US$ 1,000 million damage (Shulman, 1990) and the pesticides used to control them cost an estimated US$ 400 million (Symmons, 1992).

The desert locust can exist in two forms "solitarious" or "gregarious", which differ not only in behaviour but also in physiology and morphology. Most of the time the insects exist in the solitarious phase, drably coloured and solitary in behaviour. However, if conditions allow, the population can rapidly increase and the newly emerged hoppers have very different characteristics. They are brightly coloured in yellow and black, actively seek out each others company and start to travel in large bands. Though the change from solitary to gregarious behaviour is very rapid (it can take as little as an hour and is initiated by crowded conditions), complete morphological and physiological gregarisation can take several generations (Roessingh and Simpson, 1994; Tickell, 1996). The destructiveness of these insects is compounded by their ability to migrate (the adult swarms may travel up to 1000km a week). The environmental triggers that cause this polymorphic switch are still unknown, but rain after a period of drought is thought to be one of the major factors that cause the population increase necessary for

gregarisation. Rain provides soft soil for egg laying and a plentiful supply of fresh vegetation for the newly hatched nymphs (Haskele, 1992). It is this capacity to change both their morphology and behaviour that distinguishes locusts from other grasshoppers. The reasons for the end of plagues are even more mysterious than the factors that start them, though catastrophes such as the mass transatlantic migrations at the end of the last plague are contributory (Symmons, 1992).

The area which these gregarious desert locusts can invade is vast, affecting 57 countries and more than 20% of the total land surface of the world. When in recession, the area covered is half this and the insects in low density and the solitary phase are not a problem.

1.2 Detection of swarms and hopper bands

In order to fight these plagues it is necessary to locate swarms. Adult flying locust swarms are usually detected by land and aircraft based radar. Hopper bands of juveniles are much harder to detect and require observers on the ground or low flying aircraft, but even with these labour intensive methods it is estimated that only one in ten bands are spotted.

In an attempt to improve detection the FAO have set up ARTEMIS (Africa Real Time Environmental Monitoring using Imaging Satellites) in collaboration with NASA, National Aerospace Lab. (Netherlands) and the University of Reading. By monitoring both the real-time weather and near real-time vegetation patterns possible problem areas can be identified. In this way information about potential locust breeding sites can be relayed within a couple of days via a dedicated satellite, DIANA (Direct

Information Access Network for Africa) and control efforts can be co-ordinated more quickly (Lacoste, 1992; Hielkema and Snijders, 1994).

1.3 Chemical control

The optimum strategy for locust control is to prevent plagues by controlling upsurges, but this is usually impossible due to the poor monitoring described previously. More usually methods such as swarm elimination or crop protection are resorted to.

Control is usually achieved by the application of chemical pesticides. Ultra low volume spraying (ULV) was developed for the control of locust and was the major method of pesticide application used during the last locust swarm (Symmons, 1992). This method involves the application of the pesticide in small involatile drops. It allows quick treatment of swarms with a relatively small volume of pesticide and does not require water, which can be extremely difficult to obtain in some areas. Smaller areas are treated with hand-held or vehicle-mounted ULV sprayers, bait or back-pack EC sprayers. Larger areas are treated using aeroplanes.

One of the main techniques for controlling hopper bands is barrier spraying with a persistent chemical insecticide. This method has been tried with a number of insecticides including dieldrin and diflubenzuron. The insecticide is applied in ULV to the vegetation in swaths at right angles to the direction in which the hopper band is travelling (usually downwind) and is then consumed as the locusts travel through the effected area. The width of the band, persistence of the insecticide, ability of the insects to detoxify the insecticide, amount of vegetation and speed of travel of the hopper bands can all affect the degree of control obtained with this method (Bouaichi *et al.*, 1994a).

Many different insecticides have been applied over the last century in an attempt to control locust. Pesticide use in the early 1900s for locust control was ill-conceived, involving the use of aircraft to spray large areas with highly toxic substances such as calcium arsenate and arsenite of soda. Subsequently, persistent and cumulative organochlorine insecticides such as dieldrin were used. Dieldrin had added benefits of changing locust behaviour, the marching hopper bands slowed down and neglected to roost, often being killed by the heat of the midday sun. These chemicals are now banned in many countries due to their adverse effects on the environment and consequently are no longer produced. It has been reported that dieldrin was responsible for ending a plague in the 1960s and that it was due to its subsequent banning that there was a failure to control the upsurge in desert locusts during 1986-1987 (Symmons, 1992).

Insecticides used to control the last locust plague were the organophosphates- fenitrothion, malathion, chlorpyrifos and phoxim; the carbamates- carbaryl, bendiocarb and propoxur and the pyrethroids- deltamethrin and lambdacyhalothrin. However, these are all broad spectrum insecticides and may have detrimental effects on other organisms (Symmons, 1992).

Another group of pesticides currently undergoing field trials for use against locust are the insect growth regulators (IGRs) such as benzoyl phenyl ureas and neem insecticides. Such insecticides can be effective not only in killing insects but also at lower doses causing reduced feeding, a decrease in mobility (Grosscurt and Jongsma, 1987; Clarke and Jewess, 1990b) and egg sterility (Grosscurt and Jongsma, 1987). Acyl ureas are persistent insecticides which inhibit chitin synthesis, but unlike dieldrin they are not

cumulative so a sufficient dose to kill the insects must be applied at one time. These insecticides can be very effective, almost total control of *S. gregaria* using the benzoyl phenyl urea (BPU), teflubenzuron, has been reported (Lecoq *et al.*, 1988). Neem oil, isolated from the seeds of the neem tree, has strong phagorepellent and solitarisation affects on the desert locust. It also has the advantage of being extremely selective, even to differing degrees amongst locust species. It leads to the highest mortality on the desert locust but has a much milder effect on the migratory locust (*Locusta migratoria*) (Schmutterer *et al.*, 1993). The major disadvantage of such insecticides are that it can be several days before their effects are seen.

Spending on locust control is usually concentrated on buying pesticides, with very little money being given to studying alternatives. A total of DM 700 million was spent by the African locust control program during three years. A large proportion of this was allocated to pesticide purchase (despite the fact the probability of a locust outbreak was estimated at only 5%) and a mere 1.5% of this money was spent on researching non-chemical methods of control (Farah, 1994).

Pesticides donated for emergency locust control have contributed greatly to surpluses of outdated pesticide stocks (about 30,000-60,000 tons) which must now be disposed of at a cost of $10 per kilogram.

Pesticide use in Africa at the moment is comparatively low (it is thought to account for only 2.5% of the world imports). However, it is growing at an alarming rate, (there was an estimated 200% increase between 1988 and 1993). Legislation for the importation, formulation, distribution and use of pesticides is only now being drafted in a number of

West African countries including Benin and the legislation in Niger has only been in place for a few years (Farah, 1994).

Another problem in locust control is that during periods of recession (it can be 20-30 years between plagues) very little money is put into maintaining locust control units so that swarms are not always detected and response can be very slow. It can be very difficult to get to a remote area in which a locust swarm has broken out if there are no temporary landing strips for aircraft. Helicopters have been suggested as a better method of reaching inaccessible areas.

Some suggested methods of locust control have been very controversial. Two physicists decided that blasting swarms with lasers would be a good method of control. Later calculations, however, revealed that this was not a feasible proposition, it would take 100 laser armed helicopters 3 month to destroy a plague (Shulman, 1990)!

1.4 Biological control

Chemical methods of locust control have lead to a number of problems, such as environmental damage, high cost and low efficiency; consequently many alternative pesticides and application methods are being studied. One of these alternatives is biological control, the use of living organisms or components extracted from them, as pest control agents (Courshee, 1990).

There are a number of different strategies that can be adopted in biological control; such as the establishment of foreign predators and pathogens, the release of sterile males, the use of extracted biochemicals and the application of micro-organisms as bio-pesticides.

The migratory nature of locusts makes them very difficult to control by the introduction of predators and pathogens. The use of biochemicals, like the pheromone attractant isolated from the froth of *Schistocerca gregaria* egg pods, has so far not been widely studied (Sainin *et al.*, 1995). Most work has been done on the use of micro-organisms as bio-pesticides in particular fungi, bacteria, viruses and protozoans. Bio-pesticides in general are still very much in the research and development stage and in 1994 their sales only accounted for 4% of all pesticides sales, with a world market of $380 MM (Georgis, 1996).

Melanoplus sanguipines entomopoxvirus (MsEPV) and *Spodoptera litoralis* nuclearpolyhdrosis virus are pathogenic to a wide range of grasshoppers and locusts including *S. gregaria* (Levin *et al.*, 1993; Bensimon *et al.*, 1987). However, they have been discounted for use as insecticides due to their large host range and low virulence (Fuxa, 1993).

Isolates of the bacteria *Serratia marcescens* and *Pseudomonas aeruginosa* cause septicaemia in locust species but these also have such a wide host range (including cattle and humans) that their use is impracticable. To date no Acridid-active *Bacillus thuringiensis* strains have been discovered (Prior and Greathead, 1989).

There are protozoans which show potential as biological control agents (BCAs) of locusts, such as *Malamoeba locustae* and *Nosema locustae*. *N. locustae* has even been marketed in the USA as NOLO® bait, but it only acts as a chronic infection, reducing the life span and fecundity of grasshoppers and has met with limited success (Meneley and Sluss, 1988).

Fungi have so far shown the most promise as BCAs against locust and grasshoppers. One of the major advantages of fungi is that unlike all the other BCAs mentioned they do not have to be ingested to infect. They can act as contact bio-pesticides and penetrate directly through the cuticle.

Many fungi have been identified as natural pathogens of grasshoppers. *Entomophthora grylii*, for example, infects huge numbers of locust, grasshoppers and crickets and under suitable temperature and humidity conditions natural infections can kill up to 99% of a locust population. Other fungi that have shown potential include *Metarhizium*, *Beauveria*, *Paecilomyces* and *Verticillium spp.* (Prior and Greathead, 1989).

The LUBILOSA (Lutte Biologique contre les Locustes et Sauteiaux) project was set up in 1989 to investigate the potential of fungi for biological control, using the desert locust as the test organism. The programme has established that *Metarhizium flavoviride* isolate IMI 330189 is highly virulent to acridids with low activity against insects of other orders. As with other *Metarhizium spp.* it is non-pathogenic to vertebrates, though inhalation studies with rats revealed mild cytotoxicity at high doses (Sherwood *et al.*, 1994). This isolate was isolated in Niamey, Niger from *Ornithoacris cavroisi* (Orthoptera; Acrididae).

M. flavoviride is classified in the subdivision Deutromycotina or Fungi Imperfecti, as the sexual stage is unknown and it is believed that it may have lost the ability to reproduce sexually completely.

Interest in the use of *Metarhizium spp.* as a BCA began as long ago as 1879 (Metchnikoff, 1879). Interest in this fungus was superseded by the advent of synthetic

chemical pesticides and it was only in the 1970s that interest was renewed. The advantages of using *Metarhizium spp.* for insect control are its specificity, the lack of fungal resistance in insects, its adaptability to current pesticide application techniques and its non-detrimental effects on the environment. Its major disadvantages are its sensitivity to temperature, low environmental relative humidity, ultra violet radiation, fungicides and its slow speed of kill. Temperature and humidity are often quoted as the two most crucial factors affecting the ability of entomopathogenic fungi to infect their hosts (Milner and Soper, 1981). These factors are likely to be a particular problem with African locusts as the countries in which plagues occur tend to be very hot and dry with high levels of ultra violet radiation.

There are two species of *Metarhizium* which are currently being developed for use as biological control agents; *M. flavoviride* and *M. anisopliae*. *M. anisopliae* has been identified from approximately 300 species of Lepidoptera, Coleoptera, Orthoptera and Hemiptera though there is specificity among isolates. *M. flavoviride* exists as two or more pathovars, one of which shows high specificity for Acridids.

M. anisopliae is marketed in Brazil as Metaquino® and is used for control of the sugar-cane spittle bug, *Mahanarva posticata*. However, its success is generally low (40-70% control) which would be unacceptable to many farmers as uneconomic in parts of the Western world where producers face intense competition (Charnley, 1989a). It is thought that the variability in control success is due to fluctuations in temperature and humidity that limit the effectiveness of the fungus. Large agrochemical companies are starting to take an interest in *M. anisopliae*, Bayer has developed BIO 1020®, a granular preparation of dried mycelial pellets (Moore and Prior, 1993). Vertalec® and

Mycotal®, *Verticillium lecanii* products and Mycar® a *Hirsutella thompsonii* product, are other entomopathogens that have been marketed for biological control with varying degrees of success (McCoy, 1986; Charnley, 1989a). However, the global picture with myco-insecticides is not encouraging. Fungi currently make up a very small part of the pesticide commercial market, with world sales in 1994 of $2M a year for 12 different commercial products, although it is estimated that sales will have increased 3 or 4 fold by the year 2000 (Georgis, 1996; Ellar, 1996).

Different isolates of *M. anisopliae* and *M. flavoviride* show varying degrees of specificity for different insects. For example, *M. anisopliae* isolate ME1 has a broad host range infecting Coleoptera, Lepidoptera and Orthoptera, whereas *M. flavoviride* isolate IMI 330189 is highly specific for Acrididae. Specificity has both advantages and disadvantages, it means that there is minimal danger to non-target insects but also that they cannot be used to control a spectrum of pests unless a mixture of isolates is used. In locust control this specificity is highly advantageous, it means that the locusts can be controlled without killing other insects that may be beneficial.

Before *M. flavoviride* can be deployed in the field methods of mass production, formulation, retaining viability during storage as well as application methods need to be established.

Oil based formulations have been developed for *M. flavoviride* application during the LUBILOSA project (Bateman *et al.*, 1993). Oil formulation has several advantages over water based formulations. Firstly, water tends to evaporate very quickly from the spore on application leading to a reduced humidity in the micro-environment. Oil,

however, does not evaporate so readily and enables the spore to germinate at lower humidities. Secondly, *M. flavoviride* spores are very lipophilic and do not suspend easily in water without the addition of a surfactant. However, the spores will suspend readily in the highly hydrophobic oil which will stick to the locusts hydrophobic surface and spread much further than water formulation when applied to the insect cuticle. Thirdly, the conidia can be suspended in a much smaller volume of oil allowing a larger quantity of pesticide to be carried in one load (Bateman, 1991). Oil also makes the formulation applicable to ULV methods of application. Water is impracticable as the small volumes involved would evaporate very quickly, especially at the high temperatures likely to be encountered in the field. Water is also a precious commodity in those countries in which locusts are a problem. Finally, oil formulation affords some protection to harmful UV radiation (Moore *et al.*, 1993). Oil formulations are made in vegetable or mineral oils or a mixture of both.

A diphasic fermentation system has been developed for the mass production of aerial conidia of *M. flavoviride*. A large biomass is produced first in liquid media containing waste brewers yeast and sucrose. This inoculum is then applied to a solid media consisting of sterile par-boiled rice with a 35-40 % moisture content for conidiation (Jenkins and Goettel, 1997). One potential problem of this method of producing spores is that it can lead to rises in temperature of up to 15°C (Bartlett and Jaronski, 1988). Other work has shown that the temperature at which fungi are grown can influence not only the quantity of conidia produced but also the subsequent ability of those conidia to germinate (Abbas *et al.*, 1995; Morgan *et al.*, 1995). Another method of production which has been investigated is the use of absorbent cellulose cloths dipped in 2 to 3 day

old liquid culture and left to sporulate. Yields of up to 3×10^8 conidia per cm^2 have been achieved with this method. These simple methods of production will be of great value in African countries where cheap, low tech., small scale production is essential. Optimal methods for harvesting the spores are still being investigated.

Storage of biological pesticides is much more of a problem than for chemical insecticides, BCAs often have shorter shelf lives and have much more stringent storage requirements. Biological control agents are usually very susceptible to temperature. This is a particular problem in the areas where *M. flavoviride* is likely to be used where the storage facilities will be poor and the temperature at which the spores are stored may be very high. However, both the formulated and dry *M. flavoviride* conidia can be stored for long periods of time without the requirement for cold if thoroughly dehydrated first (Moore *et al.*, 1996).

As with many biocontrol agents the speed of kill is often slower than with chemical pesticides, although the infection can reduce a locusts capacity to harm the crop long before death by decreasing their activity and feeding (Moore *et al.*, 1992).

In part these limitations will have to be overcome with education and changes in attitude. Mycoinsecticides cannot match some attributes of chemical insecticides, but these limitations have to be weighed against the advantages of such systems.

So far field trials using *M. flavoviride* against locust have proved very successful in Benin, Niger and Mauritania (Perera, 1994). Also *M. flavoviride* has been used successfully to control *Zonocerus varigatus* (Dourokpindou *et al.*, 1995) and *Locusta paradalina* in Karoo, South Africa where it achieved a greater than 95% mortality in 4

out of 5 hopper bands after 14 days (Bateman *et al.*, 1994). However, there have been reports of excessively high temperatures in field trials which were thought to have lead to severely reduced control levels (Bateman, pers. comm.). During a period of research in Madagascar temperatures in direct sunlight were found to be 40°C or more for over 8 hours a day, increasing up to 55°C during mid-day (Scherer *et al.*, 1992).

One possible method of increasing the efficiency of *M. flavoviride* is to use it as part of an integrated pest management (IPM) system. Previous work at Bath has already shown that combined formulations of the acyl urea insecticide, teflubenzuron and *M. flavoviride* can act synergistically together to improve the speed and level of kill in third instar *Schistocerca gregaria* (Joshi *et al.*, 1992). It is thought that the synergism is due to the teflubenzuron weakening the insects cuticle by causing a reduction in the amount of chitin present and this allows hyphae to penetrate more easily.

Other problems may occur with legislation in some countries as the popularity of biological control increases. Many countries will not allow the release of introduced species. This is not a problem for the use of 330189 in Niger, its country of isolation, although it may be a problem for its use in other countries.

1.5 *Metarhizium spp.* infection

The first event in any infection is host location. For most entomopathogenic fungi, including *M. flavoviride*, this is thought to be a random event (Charnley, 1989a). Infection then consists of four major stages; attachment, germination, appressorium formation and penetration.

The mechanism of attachment for dry spores like *Metarhizium spp.* conidia are not known, but are probably caused by hydrophobic interactions between the host cuticle and bundles of rodlets on the fungal spore (Boucias *et al.*, 1988) as well as chemostatic forces. It has also been suggested that glycoproteins, enzymes and lectins could be involved in more specific attachment although as yet there is no evidence for this (Fargues, 1984). Attachment is an important factor in pathogenicity as is illustrated by the fact that very few conidia of a hypovirulent mutant of *M. anisopliae* bound to the perispiracular valves of mosquitoes compared with the virulent wild type (Al-Aidroos and Roberts, 1978).

Germination is initiated by a pre-swelling of the conidium (Dillon and Charnley, 1986) and then requires an exogenous nutrient source for germ tube formation (Dillon and Charnley, 1985). This may be a non-specific source in the case of pathogenic isolates with a wide host range, such as *M. anisopliae* isolate ME1, but more specific in those with a limited host range like *M. flavoviride* isolate IMI 330189. It has been shown that there are host related triggers associated with germination (St. Leger *et al.*, 1994a).

It is thought that the nutrient limiting conditions *Metarhizium spp.* are exposed to when they land on the cuticle may be one of the key signals for the switch from saprophytic to pathogenic growth.

In addition to being able to utilise the nutrient sources available, the fungus must be able also to tolerate any potentially toxic substances on the insect cuticle, such as the anti-fungal fatty acids of the corn-ear worm (Smith and Grula, 1982). However, it has been questioned whether these compounds are present in high enough concentrations *in vivo*

to have any effect (Charnley, 1984). Saprophytic flora may also inhibit infection. *M. anisopliae*, for example, shows enhanced germination on sterilised *Hylobius pales* cuticle (Schabel, 1978). Spore germination is highly dependent on temperature, environmental humidity, light conditions, air movement, nutrient availability and host physiological status (Milner and Soper, 1981).

Once germination has occurred the fungus starts to produce infection structures known as appressoria. These are swellings formed at the end of germ tubes. Their primary functions are thought to be attachment, the production of penetration pegs and enzyme secretion to initiate cuticle breakdown (Charnley, 1984; St. Leger *et al.*, 1986a). The formation of appressoria is central in causing *M. flavoviride* infection.

Appressorial formation in *M. anisopliae* isolate ME1 can be induced in culture by providing a hard surface and low levels of a complex nitrogenous source. High levels will inhibit appressorial formation (St. Leger *et al.*, 1989). In most cases the presence of carbon compounds will also repress differentiation into appressoria (Clarkson and Charnley, 1996). Cuticle structure can also influence appressorial formation. On smooth thick surfaces such as those found on *Schistocerca gregaria*, *Calliphora vomitoria* and late fifth instar *Manduca sexta* larvae, the appressorium is formed close to the conidia, whereas on the highly convoluted cuticle found in younger fifth instar *Manduca sexta* larvae there is extensive growth and appressorial formation occurs preferentially over hair sockets (St. Leger *et al.*, 1987b; St. Leger, 1991). Similar results were found with polystyrene replicas indicating that it is a morphological trigger (St. Leger *et al.*, 1991). Thus appressoria are thought to be produced in response to

thigmotrophic and nutritional cues and mediated by the intracellular messengers, Ca^{2+} and cyclic AMP (Clarkson and Charnley, 1996).

These stages of infection are a critical period for the fungus. During this time it is exposed to the environment and particularly susceptible to extremes of temperature, humidity and UV radiation.

Penetration typically occurs where cuticle is softer, humidity is locally high and the spore is less likely to be dislodged, such as on the arthrodial membranes and mouth parts (Charnley, 1989b). Less commonly it occurs via wounds, spiracles, gut, sense organs and pore channels (David, 1967, McCauley *et al.*, 1968; Veen, 1966; Schabel, 1978).

Cuticular penetration is achieved by a combination of enzymatic and mechanical means, evidence of this is shown in scanning electron microscopy where the presence of zones of histolysis around penetration pegs indicates enzymatic degradation of the wax layer and fissures and cuticle displacements indicate mechanical force (Zacharuk, 1970). Fungal growth in the procuticle occurs in a stepwise fashion, the fungus growing rapidly between layers of cuticular lamellae to form penetration plates and then producing short, thin penetration pegs through the much harder lamellae. Penetration plates may help to produce fissures in the lamellae making it easier for the fungus to penetrate.

Penetration is thought to be one of the stages at which specificity is determined as many pathogens can cause infection when injected into the haemocoel of an otherwise non-susceptible host (Moore and Prior, 1993). Penetration is affected by the degree of

sclerotization of the cuticle, cuticle thickness, nutrient availability, the presence of recognition factors and the presence of inhibitors (St. Leger, 1991).

Penetration through the first layer of insect cuticle, the epicuticle, is thought to be purely through enzymatic degradation, as microscopy has not identified any signs of mechanical force but has revealed the presence of zones of histolysis and large quantities of a serine-protease, PR1. Penetration through the thicker procuticle however is known to be achieved from a combination of mechanical and enzymatic forces (Clarkson and Charnley, 1996).

The mechanical penetration of plant cuticle by pathogenic fungi is thought to be primarily due to turgor pressure (Money, 1995). The plant pathogen, *Magnaporthe grisea*, can generate an estimated 8 MPa of pressure within its appressoria. It is thought this pressure is generated by the mobilisation of glycogen stores. Other factors which are thought to be connected to penetration are melanisation and actin polymerisation. Melanisation has been found to reduce the permeability of fungi to low molecular weight solutes and thus further enable it to increase turgor pressure. Little is known of the mechanical penetration used by entomopathogenic fungi.

The roles of enzymes during cuticle penetration in entomopathogens have been studied most intensively in *M. anisopliae* and it is the enzyme production in this organism that is discussed below unless otherwise stated. Due to the complex nature of insect cuticle, it is thought that penetration requires the synergistic interaction of a number of different enzymes that digest the major components of insect cuticle- protein, chitin and lipid.

In culture on 1% ground insect cuticle, proteases, together with esterases, amino peptidase and carboxypeptidase are produced very early on, within the first 24 hours of inoculation (St. Leger *et al.*, 1991). *In vivo* studies showed a similar pattern, with chymoelastase and trypsin-like activities being detected after 16 hours on *Manduca sexta* larvae and N-acetylglucosaminidase being produced after 24 hours (St. Leger *et al.*, 1987b).

Endoprotease activity in *M. anisopliae* isolate ME1 is comprised of four components PR1, PR2, PR4 and a metalloprotease. PR1 is currently thought to have a particularly important role in the infection process. Evidence includes its detection in infected insects, the effects of proteinase inhibitors on the ability of the pathogen to infect and studies on protease deficient mutants (Goettel *et al.*, 1989; St. Leger, 1995). The fact that PR1-like proteases exist in many other entomopathogenic fungi; *Beauveria bassiana*, *Verticillium lecanii*, *Nomuraea rileyi* and *Aschersonia alegrodis* also suggests that they play a very important role in pathogenesis (St. Leger *et al.*, 1987c).

PR1, a chymoelastase, is the major protein produced by *M. anisopliae* during appressorial formation *in vivo* on *Manduca sexta* and *Calliphora vomitoria* (St. Leger *et al.*, 1987a) and *in vitro* on cockroach cuticle (St. Leger *et al.*, 1995). It is specifically induced by insect cuticle *in vivo* and not by any other proteinaceous substrate so far tested (Paterson *et al.*, 1994). PR1 has considerable ability to degrade cuticle and is thought to be responsible for the solubilisation of cuticle protein leaving it open to degradation by other proteinases including metalloproteinases, carboxypeptidases and

aminopeptidases. The levels of PR1 have been shown to be increased tenfold within 24 hours of contact with cuticle (Clarkson and Charnley, 1996).

Applying a specific PR1 protease inhibitor at the same time as infection leads to a significant delay in the mortality of *Manduca sexta* larvae (St. Leger *et al.*, 1988). PR1 null mutants show partial substitution of PR1 activity with other endopeptidases which might account for the delay rather than total inhibition of penetration found during this study (St. Leger, 1995).

PR2 is a trypsin-like protease that is also induced *in vitro* by insect cuticle, but unlike PR1 can be induced by other soluble and non-soluble proteinaceous substrates. Like PR1 it is repressed by nitrogen, however adding a carbon source to the media has little effect (Paterson *et al.*, 1993). PR2 is thought to be less efficient at cuticle degradation than PR1, showing only 21% of the activity; PR4, a cysteine proteinase, shows 51% efficiency (Cole *et al.*, 1993).

Carboxypeptidases are produced at the same time as PR1 and show the same carbon and nitrogen repression indicating that the two enzymes probably function together, the carboxypeptidase breaking down the proteins solubilised by PR1 (St. Leger *et al.*, 1994b).

In culture, enzymes of the proteolytic complex are followed by the production of two chitinolytic enzymes. N-acetylglucosaminodase is produced after 24 hours and chitinases appear later, about three and a half days after inoculation and are thought to be induced by the release of N-acetyl glucosamine, the chitin monomer (St. Leger *et al.*, 1986b). *In vivo* work also indicates that chitinases are produced in quantity very late, at

least 40 hours post infection (St. Leger *et al.*, 1987b; 1995). It is possible that the low initial levels of chitinase are due to the masking of the polymer by proteins in the cuticle and that induction only occurs when the proteases have exposed the chitin. Nevertheless, cuticle from insects that have been treated with the acyl urea insecticide, diflubenzuron, (which inhibits chitin synthesis) is penetrated much more rapidly. The insecticide also enhances the virulence of *M. anisopliae* against *Manduca sexta* (Hassan and Charnley, 1989). This indicates that chitin is an important barrier to infection and it has been shown that its removal allows PR1 to degrade cuticle much faster.

Lipases are not found in culture containing ground chitin until 5 days after inoculation, before which time they appear to be cell bound (Charnley, 1989a). *In vitro* studies suggest that the digestion of insect cuticle by *M. anisopliae* follows the sequence lipase - protease - chitinase (St. Leger *et al.*, 1986c).

Once inside the haemocoel, the fungus usually starts to bud off in yeast-like growth known as blastospores but it can also grow as short hyphal fragments. This switching in growth forms, known as dimorphism, is common to many pathogenic fungi, including human and plant pathogenic fungi. The purposes of this change are not clear, but it may be that the increase in surface area to volume ratio improves nutrient uptake or that the chances of overcoming the hosts immune system are increased.

Insect defence to infection takes a number of forms. The first levels of defence are the physical barriers of the integuement, the haemocoel and the intestine. The exoskeleton also has defences in the form of the deposition of oxidised phenol (melanin) in response to infection and the presence of protease inhibitors (Ashida and Brev, 1995, *Boucias et*

al., 1988). The haemolymph is the final defence against invasion. There are a number of anti-microbials in the haemolymph such as lectins, the pro-phenoloxidase activating system, complement-like factor, cecropin, defencins and attacins (Wago, 1995). There are also a number of cells within the haemolymph that can mediate protection against invasion: phagocytosis; humoral encapsulation and nodule formation in the haemolymph, with haemocytes trapping fragments of mycelia (Charnley, 1992). These defences are generally ineffective against the more virulent pathogens.

Another form of insect defence shown by some grasshoppers is behavioural fever. When infected with *M. flavoviride* some grasshoppers are able to reduce their mortality by such behaviour (Inglis *et al.*, 1996). This study was carried out in the USA and it is possible that an even greater effect would be seen in countries like Niger where the insects can bask in very hot temperatures.

The final causes of death in insects are not yet fully understood. Extensive fungal growth may disrupt the hosts physiology and compete for nutrients (Charnley, 1984; Domnas *et al*, 1974). Fungal neuromuscular toxins (destruxins) have been shown to cause paralysis and death in some insects (Samuels *et al.*, 1988). Starvation may also be a factor due to a combination of the cessation of feeding and the fungus using the insects soluble food reserves (Charnley, 1984). The cause of death is thought to be dependent on the isolate of *Metarhizium spp.* and may also influence virulence and speed of kill.

After the death of the host, under suitable conditions (high humidity and moderate temperature), the fungus grows saprophytically back through the locust cuticle and after a while white mycelium appears on the insect surface followed by the production of

green spores. These spores then break off and attach to a new host completing the infection cycle. It would seem that the hot and dry nature of the environment in which the desert locust exists make it unlikely that many of the cadavers will sporulate in the field and therefore horizontal spread in this manner is unlikely. However, mycosed locusts can exhibit positive geotropism, burrowing into soil (where it is soft enough) to die. Additionally internal sporulation has been recorded in the field. These phenomena presumably account for the secondary cycling that has been reported (Thomas and Wood, 1997).

1.6 Aims

The aims of this work were to investigate the interactions of temperature with *M. flavoviride* isolate IMI 330189 infection in *Schistocerca gregaria*. This involved three different areas of study; firstly to look at the influence of temperature on the ability of the fungus to germinate and produce appressoria both *in vitro* and *in vivo*; secondly, to investigate the effects of temperature on the interactions between the insecticide teflubenzuron and *M. flavoviride* applied to *Schistocerca gregaria* in bioassays and finally, to examine the potential roles of the heat shock proteins HSP70 and HSP90 in temperature tolerance and pathogenicity.

2. Effects of temperature on germination, growth and appressorial formation of *Metarhizium flavoviride* isolate IMI 330189 *in vitro* and *in vivo*

2.1 Introduction

There are a number of environmental factors that strongly influence the ability of entomopathogenic fungi to persist and infect their host. These include temperature, humidity, ultra violet light and air movement (Ignoffo, 1992). Other factors which can influence the efficiency of these fungi include culture age (Hall *et al.*, 1994), culture media and spore moisture (Bateman *et al.*, 1994).

In countries where locust plagues occur, the ability to withstand high temperature is particularly important. Much work has been carried out by researchers at the International Institute of Biological Control in order to improve the formulation of *Metarhizium flavoviride* so that it can withstand high storage and application temperatures. However, as yet little work has been carried out on the effects of high temperature after the fungus has been applied to the insect.

2.1.1 Temperature and pathogenic fungi *in vivo*

High temperatures inhibit the development of entomopathogens both on the insect cuticle and inside the host haemocoel. The optimum temperature for pathogenicity, development and survival of entomopathogenic fungi is typically between 20 and 30°C (McCoy *et al.*, 1988). However, temperature requirements vary from species to species, with ecological niche and even from strain to strain. Deuteromycotina such as *M. flavoviride* isolate IMI 330189 are often found in tropical and subtropical environments and optimal germination temperatures for these fungi are usually above 25°C (Samson *et al.*, 1988).

If the temperature increases above the optimum there are effects on the fungi. In many species heat acts as an inducer for various changes, such as the initiation of germination or the prolonging of the swelling phase before germ tube production. Temperature can

also cause the fungal tips to branch, allowing the fungus to explore more of its surroundings and find a less stressful environment (Markham, 1992). However, severe temperatures are often detrimental to fungi, inhibiting germination and growth, causing the hyphal tips to burst or even destroying viability altogether.

Temperature has been demonstrated to have dramatic effects on the growth rate, germination (Morgan *et al.*, 1995), appressorial formation (Magalhaes *et al.*, 1991) or development within the hosts haemocoel (Carruthers *et al.*, 1991) of a number of entomopathogens. Temperature can also affect the time it takes to kill the host and dose required (McDonald and Nolan, 1995; Shimada *et al.*, 1995). It has been stated that the temperature ranges which occur in most agro-ecosystems are not sufficiently high to adversely affect the persistence of entomopathogens and that it is only the interaction of other additional factors (such as humidity, UV radiation and chemicals) that lead to problems (Ignoffo, 1992). However, other studies would suggest that this is probably not true for a number entomopathogens (McDonald and Nolan, 1995).

2.1.2 Temperature and the storage of entomopathogens

High temperatures have also been found to reduce the storage stability of *M. flavoviride* in aqueous suspensions (Walstad *et al.*, 1970). However, if the conidia of *M. flavoviride* are thoroughly dried before storage they become highly resistant to temperature stress (Morley-Davies *et al.*, 1996). After 60 days at 50°C *M. flavoviride* 330189 maintains 73% germination rate if stored in oil or an 80% germination rate if stored as a dried powder. However, after 90 days both formulations lost almost all viability.

The spores in this instance were dried using non-indicating silica gel. Other methods of desiccation have also been employed in order to increase the storage stability of entomopathogens, for example lyophilisation has been used successfully with *Paecilomyces fumosoreus* (Rybnikar, 1995). Phosphorous pentoxide is another chemical which can be used as a desiccant to enhance the survival of fungi (Muller, 1994).

For many biological control agents the ability to withstand such severe temperatures is unnecessary, but for *M. flavoviride* it is likely to be a problem. The areas where *M. flavoviride* is going to be used often have very poor storage facilities and it has been suggested that the biological insecticide may have to withstand temperatures of 30°C or above over a period of weeks. The recommended storage temperature for dried *M. flavoviride* spores for use in the field is between 0 and 20°C, although as this may be difficult in hot countries the spores may be stored at 30°C and still maintain sufficient viability for 3 months.

During application of the fungus in the field, temperature increases further and may reach as much as 50°C for limited periods over a number of days. In addition, once applied to the insect *M. flavoviride* has to contend with the added stress of ultra violet radiation, which has been found to interact synergistically with temperature to increase the rate of conidial deterioration. These environmental factors are also thought to have profound effects on the speed of kill (Bateman and Thomas, 1996).

For the long term storage required to maintain stocks of inoculum for manufacture, very cold temperatures must be used, for example *Nomuraea rileyi* can maintain viability for 6 years if stored at -18°C (DeSilva and Loch, 1993).

2.1.3 Interactions between temperature and humidity

Temperature and humidity are the two major environmental factors thought to limit entomopathogens. At lower humidities the temperature at which a conidia will germinate can be reduced (McDonald and Nolan, 1995). This is an important consideration as temperature and humidity can be dependent on one another. However, some studies have shown that temperature and humidity are mutually independent in their effects on fungi (Ignoffo, 1992).

Formulation in oil is one method which has been used to improve the ability of *M. flavoviride* to infect insects at higher humidities and also protect in other ways, by screening out some of the harmful UV radiation.

2.1.4 Improving low water activity and temperature tolerance in *M. flavoviride*

There are a number of methods which might be employed to change *M. flavoviride* in a manner which may allow it to overcome some of the problems caused by very high temperatures. Most of the work on *Metarhizium spp.* to date has concentrated on improving its ability to withstand low humidity. Improved ability to withstand lower humidities has the advantage of increasing the range of environmental conditions under which the fungi can infect their host as well as increasing the viability of conidia which have undergone desiccation. Similar methods could be used in an attempt to increase the tolerance of *M. flavoviride* to high temperatures.

Two methods have so far been used experimentally to increase the ability of *M. anisopliae* to infect their hosts at reduced humidity. The first is the use of media containing various different carbohydrate components to produce spores that contain an increased quantity of glycerol (Hallsworth and Magan, 1994). This method can be used to produce spores which will germinate at 5% lower water activity than control spores. Another method which has been used for *M. anisopliae* is exposure of blastospores to UV and then growing these spores up in media with a low water activity (Matewele *et al.*, 1994). This method has also been used to increased the ability of the fungi to germinate and infect insects at reduced water activities and relative humidities. However, only one of the mutants showed a statistically significant decrease in its LT_{50} value at lower humidities. Both of these methods have met with limited success as they have only slightly increased the range of water activities at which the spores will germinate.

One method that has not yet been tried is subculturing entomopathogenic fungi at elevated temperatures. This may provide useful in improving the ability of *M.*

flavoviride to infect *Schistocerca gregaria* at higher temperatures. Such a system has already been tried with entomopathogenic nematodes with mixed results. *Heterohabditis bacteriophora* had an increased thermal spectrum, but lost virulence in some parts of its thermal niche. *Steinernema anomali* did not have an improved thermal niche but its virulence was increased across the entire temperature range (Grewal *et al.*, 1996). Changes in agricultural practice may also help to make the fungus more effective. For example, by spraying at night, the excessive temperatures encountered during the day can be avoided, giving the fungus a chance to get a foothold.

2.1.5 Aims

This chapter considers the importance of temperature in relation to growth, germination and appressorial formation in *M. flavoviride* both *in vitro* and *in vivo*. It also investigates the possibility of improving the ability of *M. flavoviride* to germinate at higher temperatures by subculturing.

2.2 Materials and Methods

2.2.1 Materials

Media constituents were all from Lab M unless otherwise stated. Uvitex BHT and Uvitex OB were gifts from CIBA GEIGY. Other chemicals were supplied by Sigma Chemical Company Ltd. unless otherwise stated.

2.2.2 Methods

2.2.2.1 *Metarhizium spp.* culture

All the experiments in Chapters 2 and 3 used *M. flavoviride* isolate IMI 330189 only. Isolates of *M. anisopliae* used in Chapter 4 were treated in the same way as described for *M. flavoviride* below.

2.2.2.1.1 Preparation of *M. flavoviride* stock suspensions

Stock cultures of the locust pathogenic *M. flavoviride* isolate IMI 330189 were prepared by passaging the fungus through *Schistocerca gregaria* and then re-isolating. Adult locusts were inoculated with conidia from plates of fungus obtained from liquid nitrogen stocks and maintained as described in Chapter 3. After the insects had died the cadavers were left for 5 days to sporulate at 27°C in the dark in sealed Petri dishes containing filter paper dampened with sterile distilled water. Spores were removed from the cadaver with a sterile wire loop and plated on to a selective medium (Appendix 1). The plates were incubated for 14 days at 27°C until they had sporulated. Sterile 0.04% Tween was then poured on to the plates and the conidia were dislodged using a flame sterilised glass hockey stick. The suspension was then filtered through a double layer of sterile muslin and sonicated for 10 minutes. The spores were spun at 10,000×g for 10 minutes and the supernatant was removed. The spores were then re-suspended in fresh 0.04% Tween 80. This washing step was repeated 3 times. After the final centrifugation the spores were suspended in sterile 10% glycerol, aliquoted in to 1.5ml microcentrifuge tubes and stored at -20°C.

2.2.2.1.2 Production of stock plates

Stock plates were prepared by streaking the stock suspension on to quarter strength SDA plates (Appendix 1) using a flame sterilised wire loop. The plates were then incubated for 10-14 days at 27°C. These plates were either stored at 4°C for up to 2 months or used immediately. Further subcultures were made from these plates by pouring approximately 10ml of sterile 0.04% Tween 80 on to the plates and dislodging the spores using a flame sterilised glass hockey stick. This suspension was then poured through a double layer of sterile muslin and sonicated for 10 minutes. 100µl of this suspension was then pipetted on to a fresh quarter strength SDA plate and spread using a flame sterilised glass hockey stick. After 30 minutes drying the plates were inverted and incubated at 27°C in the dark for 10-14 days before being used. Fungi were never subcultured more than this unless otherwise stated. None of the plates (or stock plates) used for temperature experiments were stored at 4°C unless otherwise stated.

2.2.2.1.3 Preparation of aqueous and oil suspensions of conidia

M. flavoviride isolate IMI 330189 suspensions were prepared by pouring about 10ml of 0.04% Tween 80 or ondina el oil onto sporulating plates of fungi. The conidia were dislodged by gently scraping the surface of the plate with a flame sterilised glass hockey stick. This suspension was then poured through a double layer of sterile muslin and sonicated for 10 minutes as described above. The concentration of conidia was calculated using a haemocytometer and dilutions were made to obtain the desired concentration.

2.2.2.2 Growth, germination and appressorial formation *in vitro*

2.2.2.2.1 Mycelial growth *in vitro*

4mm plugs were aseptically removed from 5 day old quarter strength SDA plates of *M. flavoviride* using a flame sterilised cork borer. These were then transferred, mycelial mat face up, to the centre of fresh plates of quarter strength SDA using a sterile mounted needle. The plates were then incubated at a range of temperatures and the radial growth in mm was recorded every day.

2.2.2.2.2 Germination *in vitro*

Conidial spores were harvested in Ondina el oil and diluted to a final concentration of 3 $\times 10^6$ conidia ml^{-1}. 33µl of this suspension was spread onto the surface of a 5cm diameter Petri dish containing 15 % gelatin. The Petri dishes were then incubated at various temperatures (as described in the results) in the dark and the germination determined after 24 and 48 hours. Germination was assessed using an Olympus microscope at 400× magnification. Conidia were considered to have germinated when the germ tube was longer than the conidium.

2.2.2.2.3 Appressorial formation *in vitro*

300 conidia in 0.04% Tween 80 were used to inoculate 20ml of sterile 0.0125% yeast extract media in 7cm diameter Petri dishes (St. Leger, 1984). These were then incubated at various temperatures and the number of appressoria per conidium was assessed after 48 hours.

2.2.2.3 Assessment of germination and appressorial formation *in vivo*

2.2.2.3.1 Germination *in vivo*

Spores were prepared in 0.04% Tween 80 as described in 2.2.2.1.3 and then spun down at 4000×g for 10 minutes. The supernatant was removed. The spores were stained by re-suspending in 0.05% solution of Uvitex BHT in sterile 0.04% Tween 80 for 30 minutes. They were then applied to the insects by dipping them for 5 seconds in a solution containing 3×10^6 conidia ml^{-1}. Insects were incubated as described in Chapter 3 except that the humidity was maintained at approximately 100%. After the relevant time period locusts were killed by freezing at -20°C and different exoskeleton parts dissected out. The sections were examined with an Olympus BH-2 microscope under UV light with an L435 suppression filter at 200× magnification. Germination was assessed under a variety of different conditions as discussed in the results.

2.2.2.3.2 Appressorial formation *in vivo*

Third instar *Schistocerca gregaria* were inoculated with Uvitex BHT spores as described above and then incubated for 30 hours. The ventral abdomen was then

dissected from the insect and the number of appressoria per field of view was assessed. For each replicate 40 fields of view were counted.

2.2.2.3.3 Temperature regime and germination *in vivo*

Dead second instar *Schistocerca gregaria* were inoculated with Uvitex BHT stained spores of *M. flavoviride* as described in 2.2.2.1.3 and placed in sterile 0.5ml micro-centrifuge tubes containing sterile 0.04% Tween 80. These were placed in a Hybaid Omni thermocycler programmed as described in Figure 2-9 in the results section. Insects were prepared for germination counts by removing cadavers and squashing them on microscope slides under coverslips.

2.3 Results

2.3.1 Mycelial Growth *in vitro*

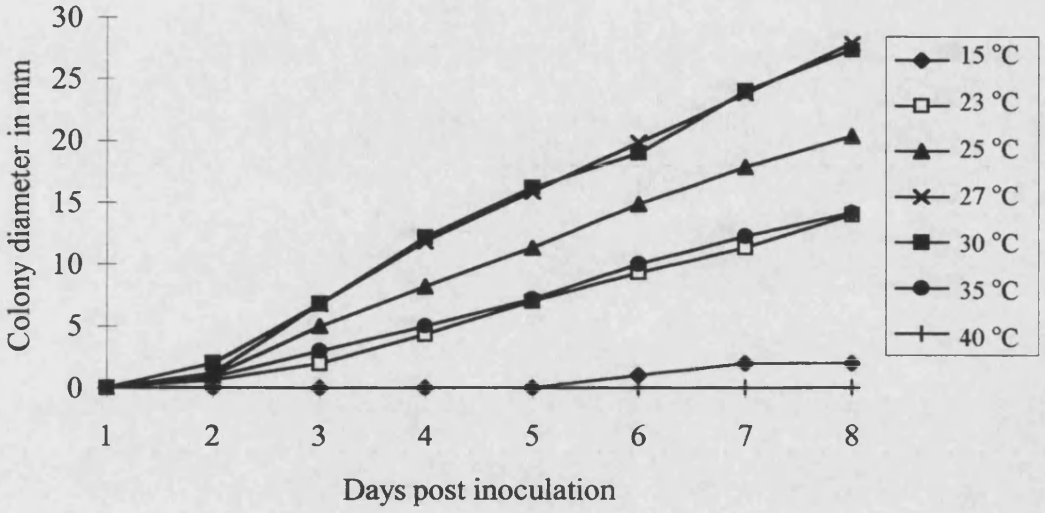
Figure 2-1 shows the results of experiments carried out to look at the ability of *M. flavoviride* mycelia to grow at a range of temperatures. At all temperatures a lag phase was initially seen when there was little or no growth, this was due to the method of inoculation. Plugs were placed on to the agar plates with the mycelial mat on the top to avoid contaminating the media. The lag phase was due to the time it took the mycelia to grow down the sides of the agar plug and reach the fresh agar media.

Of the temperatures investigated, radial growth was greatest at 27 and 30°C, the rate of growth after the lag phase being about 2mm day^{-1} under these conditions. At 25°C the rate of growth decreased by approximately 30% and at 23 and 35°C was reduced to about 50% of the growth rate of mycelia at 27°C. At 15°C growth was almost negligible and at 40°C there was no growth at all. There was very little variation in the growth rate within each treatment. Figure 2-2 shows the rate of growth in relation to temperature.

2.3.2 Germination *in vitro*

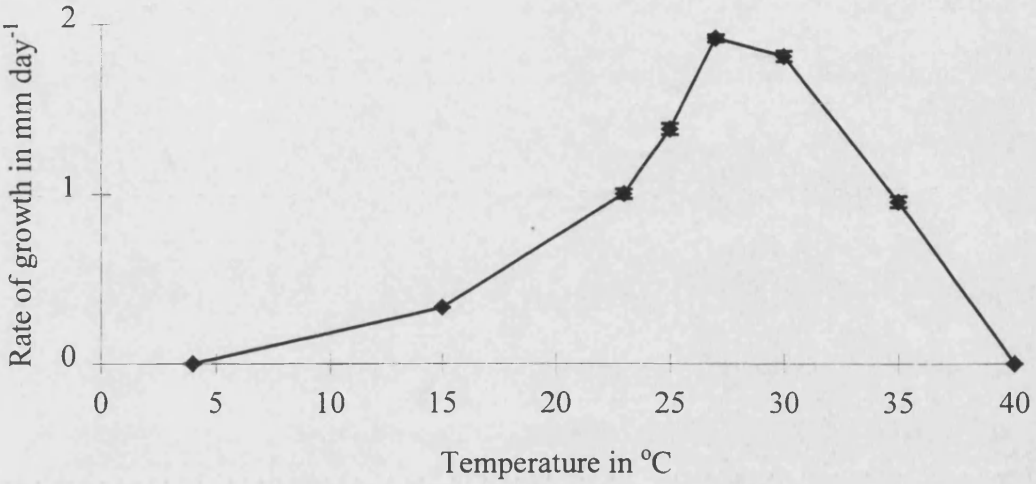
Further experiments were carried out to look at the effects of temperature on germination in *M. flavoviride*. Conidia were formulated in ondina el oil and applied to the surface of a gelatin plate. These were exposed to 45°C for between 0 and 8 hours before being moved to 27°C for a combined incubation period of 24 hours. It was found that the duration of exposure to 45°C was inversely proportional to the number of germinating conidia (Figure 2-3). For every additional two hours exposure there was a significant decrease in the number of conidia germinating after 24 hours. However, after 48 hours all treatments were found to have reached between 98 and 100% germination (data not shown).

Figure 2-1. Effect of temperature on radial growth of mycelia from a plug of *Metarhizium flavoviride* isolate IMI 330189 on quarter strength SDA plate over 8 days.



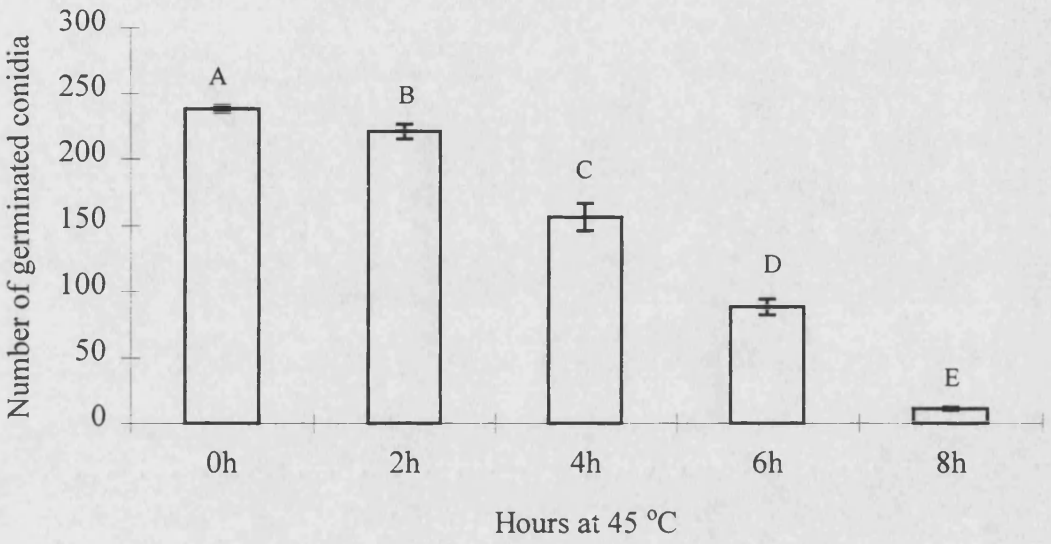
Each data point represents the mean of 6 replicates. The experiment was repeated with essentially similar results.

Figure 2-2. The effects of temperature on the rate of radial growth of *Metarhizium flavoviride* isolate IMI 330189.



Graph shows rate of growth of *M. flavoviride* on quarter strength SDA in the dark. Each point represents the mean daily radial growth of 6 replicates, bars represent +/- standard error of the mean. The experiment was repeated with essentially similar results.

Figure 2-3 The effects of duration at 45°C on the ability of *Metarhizium flavoviride* isolate IMI 330189 conidia to germinate.



M. flavoviride conidia were formulated in ondina oil and 4000 conidia were spread onto each gelatin plate. The plates were incubated at 45°C for between 0 and 8 hours before being incubated at 27°C for a combined incubation period of 24 hours. The number of germinating conidia were counted (from a total of 300 conidia). Data represents 6 replicates, the experiment was repeated with similar results both times. The bars show+/- standard error of the mean. ANOVA and student t-tests were performed on the data and letters illustrate groups which are significantly different from each other.

A preliminary experiment was also carried out to investigate the effects of exposing conidia for 8 hours to temperatures between 25 and 45°C before returning the fungus to 27°C for the remaining 16 hours of incubation (results not shown). The results showed that the percentage germination and germ tube length both correlated with the rate of growth of the fungus as shown in Figure 2-2.

2.3.3 Germination *in vivo*

Having established some of the interactions between temperature and *M. flavoviride* isolate IMI 330189 *in vitro* it was decided to investigate these interactions *in vivo*. Previous investigators have looked at germination of entomopathgenic fungi *in vivo* using aqueous formulations by using fluorescent dyes which do not affect viability (Butt, 1987). During this investigation an attempt was made to develop a method of staining spores which were suspended in oil formulation. No published protocols for this were found.

Two optical brightners supplied by CIBA-GEIGY were studied; the water soluble Uvitex BHT and the oil soluble Uvitex OB. Initial studies using methods described by Butt (1987) established that aqueous suspensions of 0.05% Uvitex BHT stained the conidia and germ tubes very efficiently. However, ondina el oil formulations of conidia containing Uvitex OB did not stain conidia, prior to or after germination on water agar. Increasing the concentration of Uvitex OB up to 200 fold made no difference.

Due to the problems of staining spores in oil formulation, further *in vivo* studies were all carried out in aqueous suspensions stained with Uvitex BHT. Before using the formulation *in vivo*, the toxicity of the formulation to both the fungi and insects was investigated. Table 2-1 shows that the Uvitex formulation proved to be non-toxic to spores at concentrations of up to 0.5%. However, a 10% solution was found to totally inhibit germination, but this was a 200 fold greater concentration than that used in the final formulation. Dipping third instar *Schistocerca gregaria* (n=30) into 0.05% Uvitex

BHT in aqueous solution caused no mortality up to 10 days after treatment (after which time the experiment was discontinued).

Third instar locusts were dipped into aqueous suspensions of 3×10^6 *M. flavoviride* IMI 330189 conidia containing 0.05% Uvitex BHT. These insects were then maintained at 25, 30 or 35°C at 100% humidity with 16h:8h light:dark cycle. These temperatures were chosen so that results could be related to information obtained from the bioassays described in Chapter 3. Germinating spores became progressively harder to count over time, possibly due to fading or due to the production of new unstained wall material.

Counts of germination were made after 12 and 16 hours, the results are shown in Figure 2-4. In almost all cases it was found that there was significantly less germination on the cuticle of insects maintained at 35°C than there was at 25 and 30°C. There was very little difference in the ability of the conidia to germinate on insect cuticle at 25 or 30°C.

When the differences between germination on different areas of cuticle were analysed it was found that there were differences in germination on all three areas of cuticle, although these were not always significantly different.

There was significantly less germination on the pronotum compared to the ventral surface of the abdomen after 12 hours, although with time as the number of germinating conidia increased these differences became insignificant.

A significant difference in the germination on the ventral and dorsal abdomen was only detected at 25°C. In general it appears that maximum germination occurred on the ventral and dorsal abdomen surfaces, with significantly less germination on the pronotum.

A similar experiment was carried out on adults. However the undulatory surface architecture of the adult cuticle made assessment of germination difficult in certain areas. Therefore, on the adult insects spores were counted on the fore and hindwing and on the ventral abdomen.

During these experiments an assessment of the distribution of spores on these different parts of the cuticle was also made. Large numbers of spores attached to the forewing and ventral abdomen (approximately 40 per field of view) whereas the inner hindwing had about ten fold fewer spores per field of view. Virtually no spores were recorded on the dorsal abdomen.

The amount of germination detected at 30°C on the ventral abdomen of adult insects (Figure 2-5) was very similar to that found on the ventral abdomen of third instar insects (Figure 2-4). However the highest percentage of germination in adults was detected on the hindwing, with slightly less germination on the forewing. It appears that both the fore and hindwings support a significantly greater degree of germination than the ventral abdomen surface.

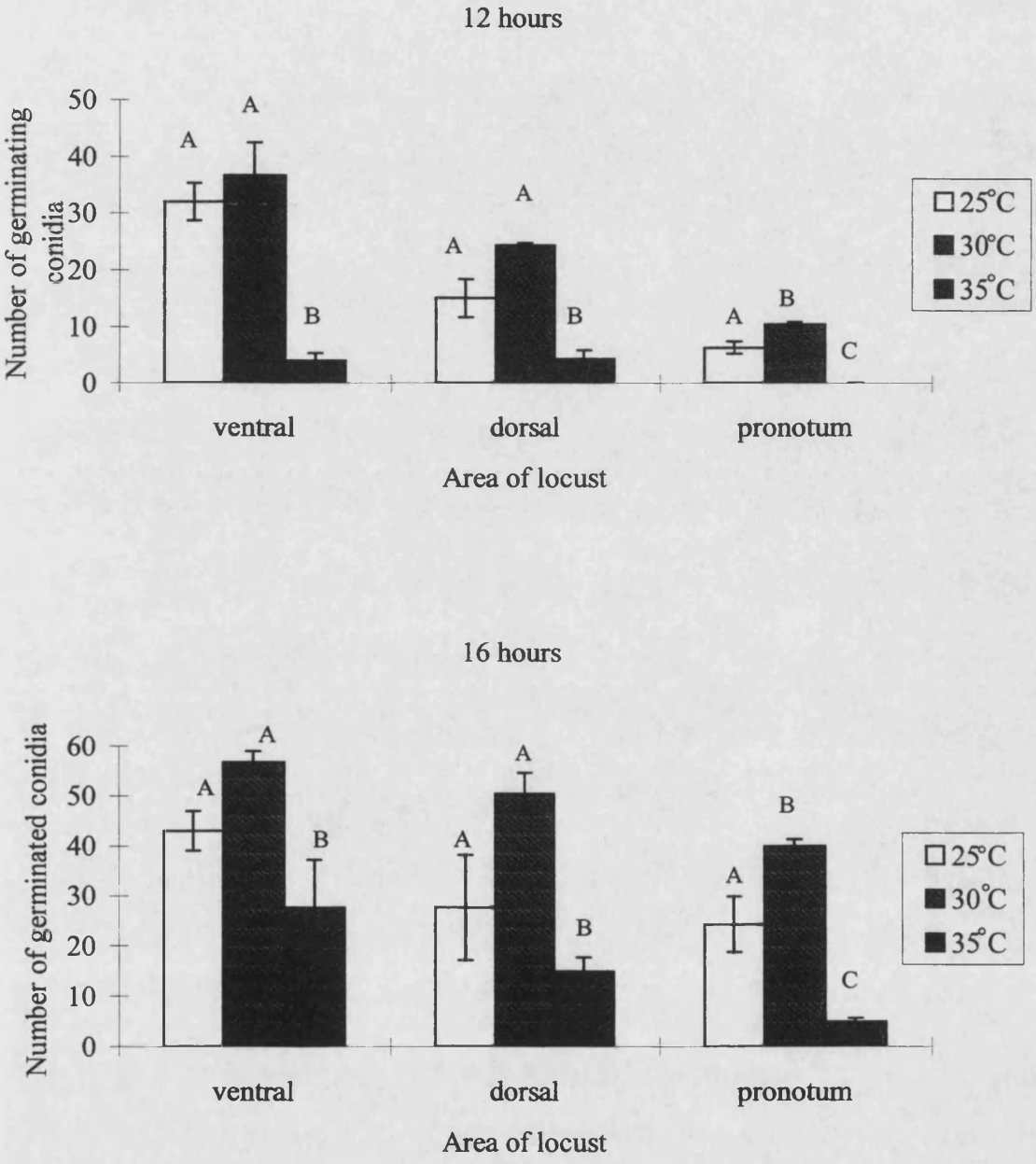
Due to the quantity of germination on the hind wing, the ease of counting spores on this area of the cuticle and the ability to compare this data with previous studies, the adult hind wing was also chosen as suitable for investigation of the influence of live and dead insects on germinating spores on the wing. Adult locusts were used and either inoculated live or first killed and then inoculated. Both live and dead insects were then incubated as described previously in containers at 27°C with a 16:8h light:dark cycle and 100% humidity. Figure 2-6 shows the results of one of these experiments. Student t-tests revealed that there were significantly more conidia germinating on dead than live locusts after 18 hours incubation (α=0.05).

Table 2-1 The effect of Uvitex BHT concentration on the ability of *M. flavoviride* isolate IMI 330189 to germinate.

% concentration of Uvitex BHT (v/v)	% germination (±s.e.)
0	99.0 (0.35)
0.05	98.5 (0.50)
0.1	98.0 (0.00)
0.5	99.0 (0.35)
10	0.00 (0.00)

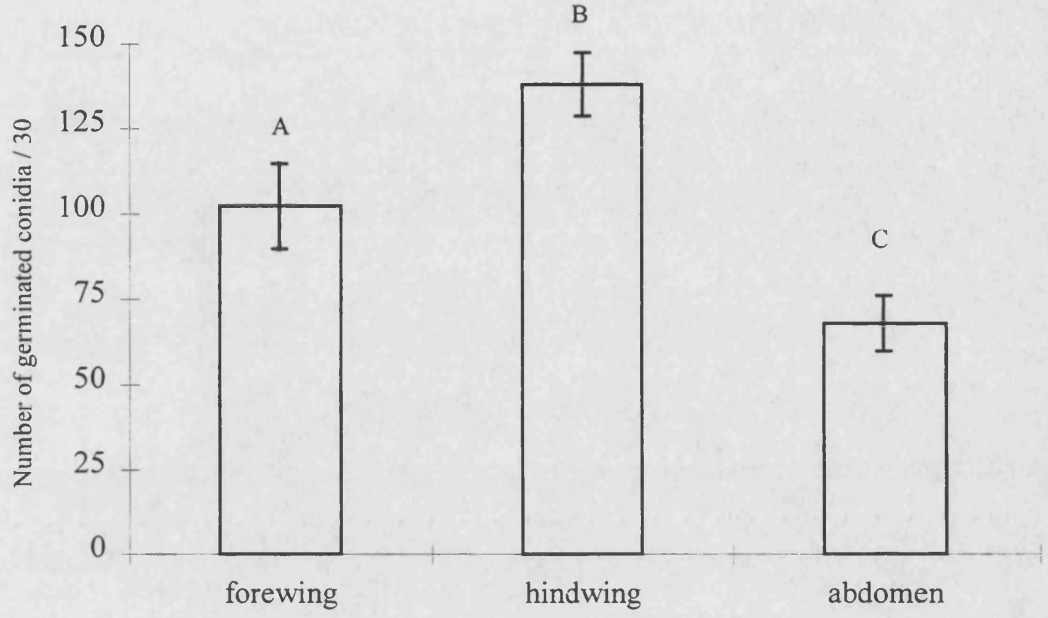
Data represents the mean percentage of germinating conidia on gelatin plates after 24 hours incubation at 27°C in the dark. Figures in brackets indicate +/- standard error of the mean. Experiment was repeated twice with similar results each time. ANOVA and student t-tests show there are no significant differences in the germination of conidia suspended in 0 - 0.5% Uvitex BHT.

Figure 2-4 Germination of *Metarhizium flavoviride* isolate IMI 331089 *in vivo* on third instar *Schistocerca gregaria* at 25, 30 and 35°C.



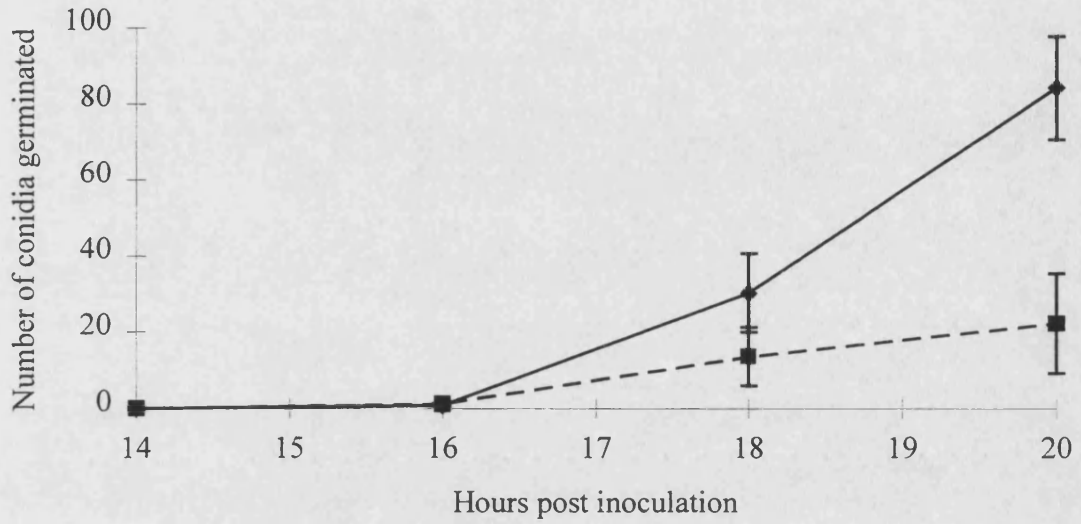
Graphs show the mean number of germinating conidia (from a total of 300) from 5 replicates, standard error of the mean is represented by the bar. Areas of the cuticle from which the germination was determined are denoted by: **ventral**, cuticle from the ventral surface of the abdomen; **dorsal**, cuticle from the dorsal surface of the abdomen and **pronotum,** cuticle from the pronotal shield. ANOVA and student t-tests (α=0.05) were carried out to compare the germination at different temperatures and on different areas of the cuticle, letters indicate groups for each area of cuticle which are significantly different.

Figure 2-5 Germination of *Metarhizium flavoviride* isolate IMI 331089 *in vivo* on different cuticle parts of adult *Schistocerca gregaria* at 30°C after 16 hours.



Bars represent the mean number of germinating conidia counted (from a total of 300) with standard error (3 replicates per treatment). The experiment was repeated with essentially similar results. Letters show groups which were shown to be significantly different using the student t-test at the α=0.05 level.

Figure 2-6 Germination of Uvitex BHT stained conidia of *Metarhizium flavoviride* isolate IMI 330189 in aqueous formulation on the forewing of live and dead adult *Schistocerca gregaria*.



Graph illustrates the number of germinating conidia from a total of 200 counted; germinating conidia counted on dead locusts are indicated by a solid line and those counted on live locusts are indicated by the broken line. Bars illustrate +/- standard error of the mean. Each data point represents the mean of 6 insects. The experiment was repeated twice with similar results each time.

2.3.4 Appressorial formation *in vivo*

The number of appressoria per field of view was assessed after 30 hours on the ventral abdomen of third instar *Schistocerca gregaria* (Figure 2-7). It was found that although on average there were less appressoria formed at 25°C than 30°C, the differences were not significant due to the high degree of variation in the data. However, there were significantly less appressoria formed at 35°C than at either 25 or 30°C.

2.3.5 Effects of temperature regime on germination *in vivo*.

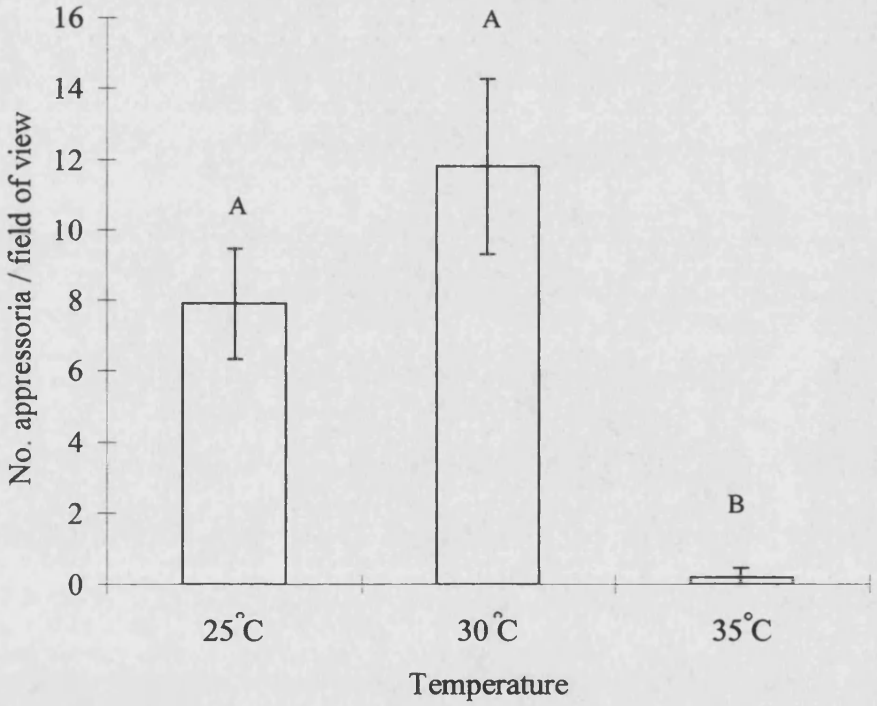
In order to look at the effects of a more realistic temperature regime on the ability of the conidia to germinate experiments were carried out using the Hybaid Omni Thermocycler. This allowed the programming of a range of different temperatures over a period of 24 hours. The variable temperature regime described in Figure 2-9 is based on information of temperature fluctuations during a brown locust swarm in S. Africa (Bateman *et al.*, 1994).

The results shown in Figure 2-8 are from experiments in which dead second instar locusts were kept in 0.5ml microcentrifuge tubes suspended in 0.04% Tween to ensure that the temperature of the insect was the same as that of the machine. The results show that after 24 hours there was a much greater percentage germination of conidia on insects which were kept at a steady 27°C than on those which were kept under the variable temperature regime. In addition under both regimes there was a significant increase in germination after a further 24 hours. After this time there was no increase in the germination in either of the two treatments.

It was interesting to note that during the entire 3 day course of the experiment no appressoria were seen in either treatment. This could be due to the method of the experiment which resulted in a large quantity of bacterial growth. These bacteria may have inhibited the growth of the fungi. Ideally, this experiment should be repeated in a dry environment to avoid this problem.

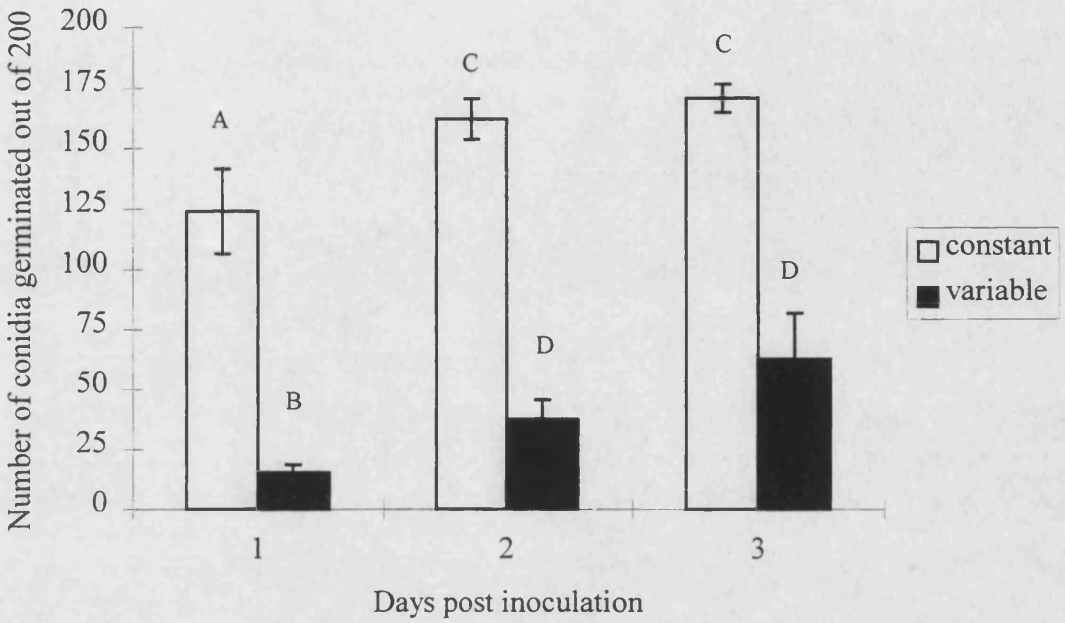
Also, these locusts an developed an exceptionally bright pink pigmentation during the experiment (some degree of change in colouration is normal during the course of infection).

Figure 2-7 The effects of temperature on appressorial formation in *Metarhizium flavoviride* isolate IMI 330189 *in vivo* on the dorsal abdomen of third instar *Schistocerca gregaria*.



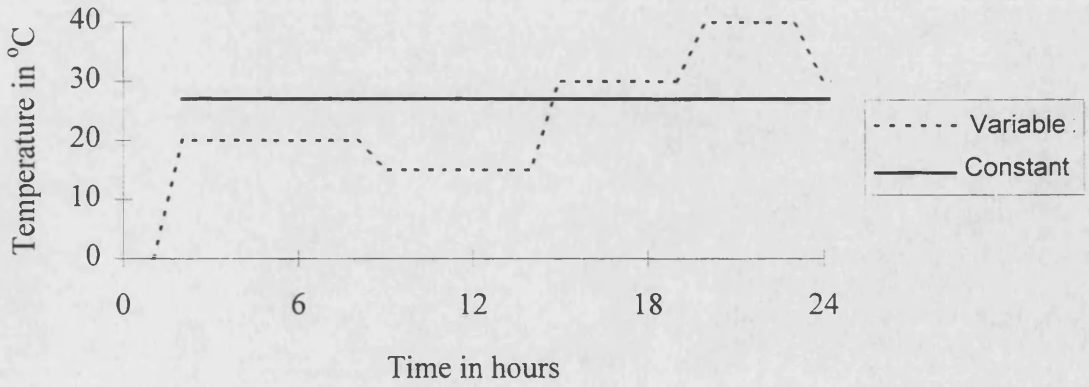
Bar chart represents the mean number of appressoria counted per field of view (area = approximately 1 mm^2) after 30 hours on adult insects kept at different temperatures as described in the methods. The bars represent +/- standard error of the mean. 40 counts were made for each of 9 replicates. Data was analysed using ANOVA and student t-tests, letters indicate groups which are significantly different to each other (α=0.05).

Figure 2-8 The effect of variable and constant temperature regimes on the germination of *Metarhizium flavoviride* isolate IMI 330189 *in vivo* on dead second instar *Schistocerca gregaria*.



Bars represent the mean number of germinating conidia from 5 replicates counted on second instar squashes 24, 48 and 72 hours after inoculation, lines show +/- standard error. Letters represent means which the student t-test showed to be significantly different to each other.

Figure 2-9 Temperature regimes to which the second instar *Schistocerca gregaria* were subjected.



2.3.6 Effects of subculturing on ability to germinate at higher temperatures.

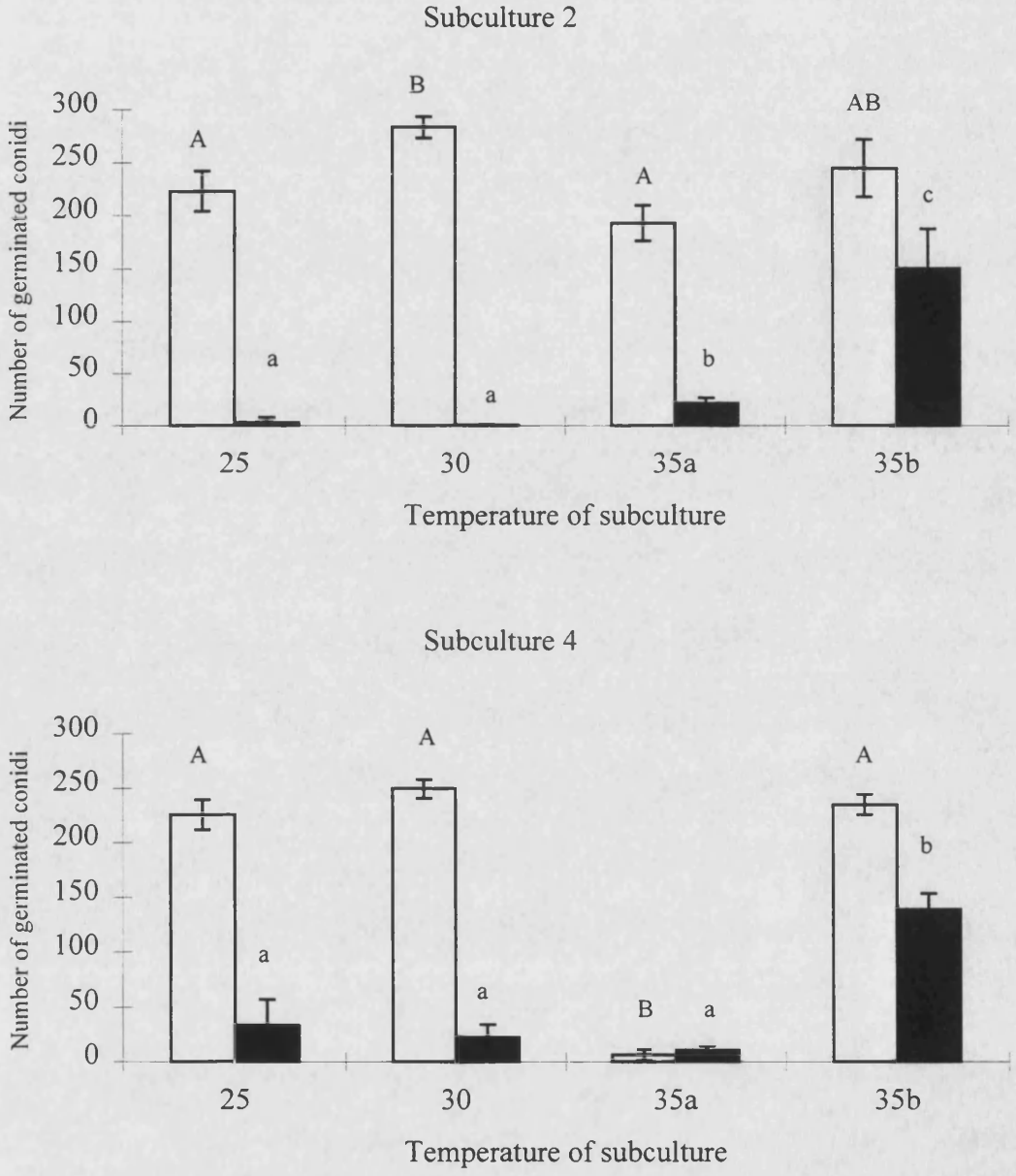
In an attempt to look for possible methods of improving the infectivity of *M. flavoviride* at higher temperatures, the effects of subculturing at different temperatures on subsequent germination at higher temperatures were observed. *M. flavoviride* was grown as described previously except that the culture temperatures used were 25, 30 and 35°C. The conidia from these plates were subcultured up to six times. The ability to germinate after 24 hours at 27°C or 8 hours at 45°C followed by 16 hours at 27°C was then assessed on gelatin plates after every two subcultures.

Fungi cultured at 35°C took significantly longer to grow and sporulate than at 25°C and the difference between the two treatments became more marked with each subculture. By the sixth subculture none of the fungi subcultured at 35°C had sporulated after 40 days (Figure 2-11). Some of the fungi subcultures at 30°C also showed a reduced ability to sporulate, none of the fungi cultured at 25°C showed these symptoms.

The conidia were harvested from all plates 20 days after inoculation in order to ensure that all fungal cultures had sporulated. Figure 2-10 shows that after two subcultures germination at 27°C was significantly better in conidia from fungi which had been subcultured at 30°C than those which had been subcultured at 25°C. ANOVA showed that fungi which had been subcultured at 35°C fell into two distinct groups (refer to 35a and 35b in Figure 2-10) and therefore the data could not be pooled. After two subcultures both the groups subcultured at 35°C had significantly better germination at the higher temperature than conidia from fungi subcultured at 25 or 30°C.

After four subcultures there were no significant differences observed in the ability of the 25, 30 and 35b groups to germinate at 27°C. At the 45°C treatment however 35b showed significantly more germination than any of the other groups. 35a showed very poor germination under both the test regimes and was noted to show rather poorer growth, with many fewer conidia retrieved per plate.

Figure 2-10 Effect of subculturing *Metarhizium flavoviride* isolate IMI 330189 at different temperatures on the subsequent ability of the conidia to germinate at 27 and 45°C.



Mean number of germinating conidia from a total of 300 (± standard error) after 24 hours at 27°C (unshaded bars) or after 8 hours at 45°C followed by 16 hours at 27°C (shaded bars). Data represent 3 replicate counts made from each of 4 subcultures for each treatment. Data from 35°C subculturing could not be pooled as ANOVA revealed significant differences between groups, this data has therefore been divided into two groups which each represents 2 subcultures. Data were analysed using the student t-test and letters indicate groups were significantly different to each other (N.B. germination for each treatment was analysed separately).

Figure 2-11 Effect of repeated subculturing *Metarhizium flavoviride* at 35°C on the growth of *Metarhizium flavoviride* isolate IMI 330189

Photograph shows *M. flavoviride* isolate IMI 330189 culture which had been subcultured six times at 35°C. Plate was incubated for 40 days during which time the fungus failed to sporulate

2.4 Discussion

2.4.1 Mycelial growth *in vitro*

M. flavoviride isolate IMI 330189 mycelia was found to grow fastest at 27 - 30°C under the conditions used in this experiment, with a growth rate of about 2mm day^{-1} after the lag phase. It did not grow at all at 40°C or 4°C. At 25°C the growth rate was about 30% slower than for mycelia grown at 27°C. It is interesting to note that a similar investigation (Bye, 1993) carried out on *M. anisopliae* isolate ME1 revealed very little difference in the growth rate of mycelia at 25, 27 or 30°C, where the growth rate was at an optimum of approximately 2mm day^{-1}. However, *M. flavoviride* isolate IMI 330189 had a much faster growth rate at 35°C compared to *M. anisopliae* isolate ME1. It would appear that this isolate of *M. flavoviride* has a higher optimal growth range than *M. anisopliae* isolate ME1. This could be explained by the environments from which each strain was isolated, ME1 was isolated from the USA whereas *M. flavoviride* strain IMI 330189 was isolated from the much hotter and drier climate of Niger. It could also be due to differences in the host from which each strain was isolated. The *M. anisopliae* strain was isolated from a beetle and the *M. flavoviride* from an Acridid. Differences in behaviour of these two insects may well lead to differences in their body temperatures, which could account for the differences in requirements for growth at different temperatures for each *Metarhizium spp.* Welling *et al.*, (1994) also reported that an isolate of *M. flavoviride* could grow at higher temperatures than a number of *M. anisopliae* isolates tested. However, another report on the rate of radial growth of another isolate of *M. anisopliae* carried out in the same way has shown that the fungus grew faster at 35°C than at 27°C (Hallsworth and Magan, 1996). This illustrates that the differences in growth rate of *M. anisopliae* isolate ME1 and *M. flavoviride* isolate IMI 330189 at different temperatures are dependent more on the isolate than on the species. Samuels *et al.* (1989) tested the growth rate of a number of *M. anisopliae* isolates at different temperatures. The results showed a high degree of variation between the isolates and also that those which had the highest growth rates at 27°C were

also the most pathogenic to *Nilaparvata lugens*. This gives further evidence for the importance of temperature tolerance in pathogenicity. Other workers have also reported variations in mycelial growth of *M. anisopliae* isolates at different temperatures (Hywel-Jones and Gillespie, 1990).

2.4.2 Germination *in vitro*

With *M. flavoviride* conidia it was found that the length of exposure to 45°C was inversely proportional to the percentage germination obtained after 24 hours. However, further counts made after 48 hours showed 98-100% germination in all treatments, indicating that exposure to high temperatures merely delayed germination. It was not determined whether the length of exposure to 45°C related directly to the delay in germination, though casual observations suggested that the length of the germ tubes was inversely proportional to the length of time for which the conidia had been exposed to 45°C. Other workers have reported that the lag phase of germination in *M. anisopliae* is temperature dependent (Hywel-Jones and Gillespie, 1990). Germ tube extension in *M. anisopliae* has been found to correlate with the percentage germination (Hallsworth and Magan, 1995).

From this data it would appear that temporary exposure to high temperature could lead to a delayed kill of insects.

Such a phenomenon has been described for the plant pathogenic fungi *Penicillium expansium* (Fallik *et al.*, 1996) and *Fusarium oxysporum*. In *Fusarium oxysporum* spores exposure to high temperature caused a reduction in their ability to germinate, in their pathogenicity and in the quantity of carbon present (Arora *et al.*, 1996).

In contrast to the present work, Scherer *et al.* (1992) have shown that if *M. flavoviride* conidia are subjected to 60°C for 5 hours while in oil formulation before being applied to the gelatin plate there was no reduction in germination over this period. These data would suggest that *M. flavoviride* spores are very resistant to temperature changes while in oil formulation but become far more susceptible to temperature extremes once they

have begun the process of germination. However, the differences between the results of Scherer *et al.* and the results found in this study may also in part reflect differences in methods of fungal culture and formulation between the two experiments.

This investigation did not look at the effects of repeated exposure to high temperatures that may well occur in the field. Repeated exposure to extreme temperatures may not allow the conidia sufficient time for recovery and germination. In addition germlings may be more sensitive to temperature increases as conidia are often found to be a very resilient life stage. This is an area which will require further investigation. Potential problems of the cumulative effects of repeated exposure to high temperature have also been speculated on by McGuire *et al.*, (1987).

2.4.3 Germination *in vivo*

All *in vivo* work was carried out by staining *M. flavoviride* spores in Uvitex BHT which allowed spores to be visualized on non-transparent cuticle surfaces.

The toxicity and ability to stain spores was investigated in two Uvitex fluorescent brightner products, Uvitex BHT (water soluble) and Uvitex OB (oil soluble). Neither of the two products appeared to be toxic to *M. flavoviride* at or below 1% solution. This meant that they could be used to stain spores before they were applied to insects. The ability of aqueous Uvitex BHT to stain spores has already been documented and was found to be applicable to *M. flavoviride* 330189 (Butt, 1987). Unfortunately, spores suspended in Uvitex OB in ondina El oil did not take up the dye.

The rate of germination *in vivo* on live third instar *Schistocerca gregaria* was found to be affected by both temperature and location (Figure 2-4). Germination was greatest at 30°C for all the body parts examined. Germination was lower at 25°C (although in most cases there was no significant difference in the germination rates at 25 and 30°C) and at 35°C there was virtually no germination at all. This pattern of germination

correlates well with the *in vitro* growth rate at these temperatures described in Figure 2-2.

In third instar *Schistocerca gregaria* the greatest proportion of germination was on the ventral abdomen surface, followed by the dorsal abdomen and then the pronotum. There could be a number of reasons for these differences. The differences may be due to differences in inhibition on different areas of the cuticle. This inhibition could be caused by a number of chemical factors, such as the quantitative and qualitative variation in lipids found on different areas of *Schistocerca gregaria* (Lockley, 1970). The percentage germination is highest where the cuticle stiffness is least, on the ventral surface of the abdomen and is lowest where the stiffness is greatest, on the pronotum. It could be that some physical or chemical factor involved in cuticle stiffness is detected by the fungus and influences germination. *M. anisopliae* has previously been found to form appressoria preferentially over *Manduca sexta* hair sockets or integuemental membranes where the cuticle is thinner and is proposed to represent a zone of weakness (St. Leger *et al.*, 1988, Charnley, 1989b). The germination could also be related to differences in nutrients on each area of the cuticle (St. Leger, 1991).

Three different areas of adult locust cuticle were also inspected - the hindwings, the forewings and the ventral abdomen. Germination was greatest on the hindwing, although the mean number of spores per field of view was much lower. Germination was also high on the forewing. This pattern of results again supports the hypothesis that cuticle stiffness strongly influences germination, as in this case the proportion of germination is also highest on the softer areas of cuticle. The differences in the adult could also be due to micro-climate as the hind wing probably has a very high humidity because it is protected by the forewing. However, as the insects were maintained at 100% humidity this should not have been a factor. Other workers have found very differing rates of germination *in vivo* using similar methods, this has been put down to the effects of varying humidity (McGuire *et al.*, 1987, Magalhaes *et al.*, 1991).

Although these experiments were carried out in aqueous suspensions rather than in oil formulation they do give some indication of potential problems which may be caused by excessive temperatures. Temperatures which have been measured in the field while studying the possibility of using *M. flavoviride* as a biological control agent often reach well in excess of 35°C (Bateman *et al.*, 1994; Scherer *et al.*, 1992). In Madagascar temperatures of up to 55°C were recorded, with the temperature being over 40°C for at least 8 hours a day. Under these conditions *M. flavoviride* is unlikely to be able to infect its host. However, the temperature of the host is not necessarily the same as its environment and insects can regulate their temperatures by positioning themselves in shade or light and by body posture. But as *Melanoplus sanguinipes* have been shown to demonstrate some degree of behavioural fever leading to reduced mycosis when infected with *M. flavoviride,* these high temperatures may be even more detrimental (Inglis *et al.*, 1996).

These experiments also revealed that the rate of germination on live locust appeared to be very much slower than has been previously obtained with dissected and sterilised locust hind wings (James, 1995). In the present study, spore germination at 30°C on the hind wing of live adult locust after 12 hours was found to be so low that counts were not made until 15 hours post inoculation and by this point germination had only reached 42%. With the latter method, 88.6% germination was reported after 12 hours incubation on sterilised hind wings maintained at 27°C. Further evidence of differences in germination on live and dead insects was also presented in this investigation. Germination on the hind wing of dead locusts was found to be significantly faster than that on live locusts (Figure 2-6).

There may be a number of reasons for these differences. Firstly, conidial germination may be inhibited by anti-fungals present on the surface of the locust. A number of anti-fungal components have been recognised on insects' cuticle - fatty acids (Smith and Grula, 1982), phenols, quinones and lipids (St. Leger *et al.*, 1991). However, to date no

work has been done which conclusively shows that these chemicals are involved in disease resistance. However, an anti-fungal phenol present in the gut of *Schistocerca gregaria* has been described which is present in sufficient quantities to inhibit the germination of *M. anisopliae* (Dillon and Charnley, 1988). Secondly, the reduced germination rate may be due to the presence of micro-organisms on the surface of the locust. Enhanced germination of *M. anisopliae* has been shown on cuticle of *Hylobius pales* which has been first surface sterilised (Schabel, 1978). Also the inhibited germination described by Dillon and Charnley (1988) is thought to be due to micro-organisms as the same phenomenon is not observed in axenic locust. If this proves to be the case on the cuticle of *Schistocerca gregaria* it will lead to some problems in investigating the importance of surface micro-flora, as the micro-organisms associated with laboratory kept animals are likely to be very different to those on locusts in the field.

Finally, some of the differences may be due to decreased accuracy in counting since the mycelia did not stain as intensely as the conidia and may have been overlooked in some cases.

The implications of reduced germination at high and low temperatures are very important. The ability to germinate quickly has been associated with virulence in *Metarhizium spp.* against *Schistocerca gregaria* (James, 1995) and in *Beauveria bassiana* against the corn ear worm (Perkrul and Grula, 1979). James (1995) found that of a range of possible factors influencing the virulence of *Metarhizium spp.*, only germination showed a significant correlation to median lethal time in adult *Schistocerca gregaria.* Other studies have shown that if the speed of germination and appressorial formation in *M. anisopliae* are increased by pre-soaking, that the LT_{50} of *Manduca sexta* infected with these conidia will be reduced (Dillon, 1984). These results indicate that the significantly reduced germination rates detected on locust at 35°C in this study may

relate to a significant decrease in the virulence of *M. flavoviride* against *Schistocerca gregaria* at this temperature.

Assessments of the effects of temperature on appressorial formation revealed exposure to increased temperature had significant effects. The number of appressoria was significantly reduced in those treatments kept at 35°C, compared with treatments at 25 and 30°C. However, it is very hard to establish whether this is a entirely a factor of the delayed germination and growth at this temperature or whether it is due to a genuine reduction in the ability to form appressoria. It was evident that there was much less growth at 35°C than at 30°C. Appressorial formation has been found to be far more sensitive to environmental and nutritional changes than germination in the entomopathogen *Zoophthora radicans* (Magalhaes *et al.*, 1991; Galaini-Wraight *et al.*, 1992).

2.4.4 Improving temperature tolerance

Subculturing *M. flavoviride* at 35°C did improve the ability of the conidia to germinate at high temperatures without affecting their ability to germinate at 27°C. However, this pattern was only seen in half the subcultures, the germination ability of other conidia subcultured at 35°C declined rapidly under all conditions. It would appear that this may be a method that could be used to improve the tolerance of *M. flavoviride* to extreme temperatures, but it must be used with caution. During this investigation no assessments of the effects of subculturing on pathogenicity were made. The ability of *M. flavoviride* subcultured at 35°C to grow clearly declined over the course of the experiment, to the extent that after 6 subcultures the fungi appeared to lose the ability to sporulate. Although it is important to improve the ability of conidia to withstand higher temperatures this should not be done at a cost to other attributes.

Other workers have found that the temperature at which secondary conidia of *Erynia neoaphidis* are produced will effect their subsequent rate of germination (Morgan *et al.*, 1995). These authors found that any conidia produced at any sub-optimal temperature

had a reduced rate of germination at all the temperatures tested. The type of secondary conidia produced by this fungus was also found to be effected by temperature. Other work has shown that the temperature for optimal conidial production in a plant pathogenic fungus is the same as the temperature required for optimal germination rates, but that this was not the same as the optimal growth rate temperature (Abbas *et al.*, 1995). It was also found that the conidia which were produced at the higher temperatures which produced rapid growth had a very low infectivity and different biochemistry. Whether optimal growth rates are related to optimum spore production in *M. flavoviride* has not been investigated and there could be potential to increase production mass with this technique.

One potential problem with the subculturing experiment was that in order to ensure that conidia were taken when all Petri plates showed signs of sporulation, conidia were not harvested until plates from all temperature treatments were 20 days old. Fungi grown at 25 and 30°C sporulated several days before fungi which had been grown at 35°C. It has been shown previously that the rate of germination is influenced strongly by the age of spores in a number of entomopathogenic spores, including *M. anisopliae* (Hall *et al.*, 1994). As the spores which were harvested at 25 and 30°C were older than those harvested from 35°C it might be expected that the germination in these treatments may have been slightly reduced.

Subculturing has previously been shown at Bath to affect the pathogenicity of *Verticillium lecanii* to aphids (Bye, pers. comm.). When the fungus was passaged through 1% locust cuticle agar there was evidence of increased virulence.

There are other methods that could be used to increase the ability of *M. flavoviride* to infect locusts at higher temperatures some of which were discussed in the introduction to this chapter. Hallsworth and Magan (1995) have already demonstrated that *M. anisopliae* conidia which have been grown on media supplemented with trehalose will have increased intracellular trehalose levels. Other workers have shown that increased

trehalose levels can have a drastic effect on improving the ability of fungi to survive high temperatures (Tereshina *et al.*, 1991). However it has also been reported that increased trehalose levels in *M. anisopliae* lead to a reduced germination rate (Hallsworth and Magan, 1996). It may be that improving the infectivity of *M. flavoviride* at higher temperatures by increasing trehalose levels may lead to costs in the speed of kill which would be unacceptable. However, the same investigation also showed that increasing the levels of glycerol and polyols did increase ability of *M. anisopliae* to germinate even at reduced water availability. These chemicals have also been reported to improve thermotolerance, although their abilities to improve thermotolerance are less well documented than those of trehalose.

Other workers have found that the growth media which leads to the greatest degree of heat resistance in *Bacillus cereus* spores is dependent on the strain and hence no generalizations can be made on which media will induce greatest thermotolerance in spores (Mazas *et al.*, 1995).

Another potential method which could be used to increase the ability of *M. flavoviride* to infect at higher temperatures is mutagenesis of blastospores using UV radiation. This method has already been successfully used with *M. anisopliae* to increase the ability of this fungus to germinate and infect leafhoppers at lower water activities (Matewele *et al.*, 1994).

Finally, the possibility of using combined formulations has also been considered (Inglis *et al.*, 1996). In this case the aim was to improve the efficacy of *M. flavoviride* at lower temperatures and of *Beauveria bassiana* at higher temperatures. The results showed that combined formulations of the two entomopathogens lead to an increased mortality at higher temperatures but had no effect at lower temperatures.

3. Interactions between teflubenzuron and *Metarhzium flavoviride* isolate IMI 330189 against the desert locust *Schistocerca gregaria* and the effects of temperature.

3.1 Introduction

3.1.1 Integrated Pest Management

Metarhizium flavoviride is currently being developed as a stand-alone pesticide, although it may be more effectively used as a part of an integrated pest management (IPM) scheme. That is, *M. flavoviride* could be used in conjunction with one or more chemical or biological pesticides in order to improve its effectiveness or selectivity. In particular, combined formulations may be used to help overcome some of the temperature constraints currently experienced when using *M. flavoviride*.

The idea of using IPM is currently very popular and a global IPM initiative has recently been established to encourage the use of low chemical pest management in developing countries. This programme has been given an annual budget of US$1 million by the FAO and World Bank, but it is hoped that other organisations will also support the initiative (Pesticide News, 1995a).

One example of the development of an IPM system using entomopathogenic fungi to control locust is provided by scientists from ICIPE (International Centre for Insect Physiology and Ecology) in Nairobi. Here, they have been working on a system of IPM which involves the synergistic use of an anti-gregarisation pheromone and an entomopathogenic fungus. It appears that the pheromone weakens the physiology of the locust and makes it much more susceptible to disease and pesticides. The dose of fungus required to kill the insect is reduced ten fold if applied at the same time as the pheromone. The group have recently won the World Food Prize for their work (Radford, 1995).

The advantage of using IPM is that combined applications of pesticides can decrease the doses required (thus limiting potential environmental damage), decrease the time taken to kill a pest or increase the selectivity of an insecticide. If the combined effects of two pesticides applied together is greater than the sum of their individual effects, as in the above example, this is described as synergy.

Insects are often found to be more susceptible to pathogen attack when they are stressed by some other factor, such as malnutrition. It is possible that chemical insecticides may also act as stressors, increasing the efficiency of any microbial pathogens subsequently applied.

Previous work at Bath has shown that the benzoylphenyl urea (BPU), teflubenzuron (Nomolt™) acts synergistically in combined formulation with *M. flavoviride* isolate IMI 330189 against third instar *Schistocerca gregaria* in laboratory bioassays (Joshi *et al.*, 1992). For this experiment both insecticides were formulated in oil and applied topically. The combined formulation not only caused a significant increase in mortality but also decreased the time until death. Another BPU, diflubenzuron, has also been found to act synergistically with *M. anisopliae* used against *Manduca sexta* (Hassan and Charnley, 1983).

BPUs are insect growth regulators (IGRs) which inhibit chitin synthesis in insects causing newly laid down cuticle to be weak and brittle. The poisoned insect usually dies at the next moult either as a result of failure to ecdyse or due to a rupturing of the new cuticle which causes dehydration (Wright and Retnakatan, 1987). BPUs may aid fungal penetration of the insect by weakening the cuticle and in the absence of chitin make the protein more accessible to degradation by proteases like PR1.

It is important to take into account the effects of potential synergistic pesticides on *Metarhizium* growth and sporulation. Insecticidal BPUs have been shown to have no effect on fungal chitin synthesis. Initial studies have shown that teflubenzuron does not affect conidial survival in *Metarhizium spp.* even with prolonged storage (Joshi, 1993)

but other pesticides have been found to have adverse effects on *Metarhizium spp.* (Li and Holdom, 1994).

Before discussing the possible interactions of teflubenzuron and *M. flavoviride* it is necessary first to understand a little of the structure of insect cuticle and how teflubenzuron can influence it.

3.1.2 Chitin in insect cuticle

Cuticle provides insects with an exoskeleton, prevents water loss and provides skeletal muscle support. It is also acts as the insects primary defense against pathogen and predator attack. It is because of the effectiveness of this protective layer that the majority of insect pathogens (viruses, amoebae and bacteria) must be consumed to cause infection.

Insect cuticle consists of two layers. The thin outer epicuticle contains phenol-stabilised proteins and is covered by a waxy layer which is secreted by pore canals. This wax layer is in turn covered by a layer of cement which is placed on the surface during a moult. The inner layer consist of a much thicker procuticle which mainly contains chitin fibrils embedded in a protein matrix. The procuticle can be divided into two layers in most insects, the sclerotised outer exo- (or pre-ecdysal) cuticle and the inner endo- (or post-ecdysal) cuticle. Sometimes a third layer can be differentiated between the exo- and endocuticle and this is known as the mesocuticle. These cuticle layers are formed by the epidermis which in turn is attached to the basement membrane (Reynolds, 1987).

Most of the physical properties of cuticle are determined by the procuticle. In locusts the cuticle is very rigid apart from the arthroidal membranes present at the insects joints. This means that insects must periodically under go ecdysis, shedding its old cuticle to reveal a new softer cuticle which hardens over time through the cross-linking of

proteins. Insects are very vulnerable during the moult. Gregarious desert locusts go through this cycle five times during their life time.

Ecdysis occurs in a number of stages. Firstly, the old layer of cuticle becomes detached from the epidermis (apolysis). Next there is a re-organisation of the epidermis which may involve cell proliferation. The insect then starts to deposits a new layer of cuticle under the old layer. After a while enzymes in the moulting fluid between the two layers of cuticle begin to digest the old procuticle. Finally, the moulting fluid is reabsorbed before insect sheds the remainder of its old exoskeleton.

Insect cuticle is composed of protein, chitin, lipids, catechols, water, minerals and pigments. Chitin is the second largest component in most insect cuticles accounting for between 20-60% of the dry weight, although it is usually only found in the procuticle (Anderson, 1979; Hackman and Goldberg., 1987). The procuticle is mostly made up of chitin fibrils embedded in a protein matrix. Up to 70% of this layer is protein (locust cuticle has been shown to contain over 100 different proteins), with sheets of chitin laid down in a helicoidal arrangement giving rise to a lamellate structure. The more pliant cuticle is the more chitin it is likely to contain (Hillerton, 1984).

Chitin is a viscoelastic polymer with stiffness, strength and extensibility (Hepburn, 1985). It is a polymer of 2-acetylamido-2-deoxy-D-glucose monomers joined by β-1,4 - glucosidic linkages. Three crystalline forms of chitin exist- α, β and γ; but only the α form is found in insect cuticle (where chitin polymers are antiparallel in sheets). This is the most stable of the chitin forms (Rudall and Ketchington, 1973). All three chitin types can exist in the peritrophic membrane of insect guts.

3.1.3 Insecticides which effect the chitin content of insect cuticle

Chitin is a good target for insecticides as it is universal in insects (and other arthropods) and relatively uncommon in other animals (except for fungi and some diatoms). Many insecticides operate by decreasing the amount of chitin in insect cuticle, leading to the effects described earlier. These insecticides include nucleoside peptides, sulfhydryl

reactive agents and many insect growth regulators (benzimidazoles, triflumuron, buprofezin, phenyl carbamides, plumbagin and benzoylphenyl ureas). All those which have been described to date act on the polymerising step of chitin production.

As stated earlier benzoylphenyl ureas are among these insecticides. They were first discovered in the 1970s by Philips-Duphar whilst looking for a more effective weed killer. Poisoning symptoms in the insects on the dosed plants were observed after a few days when the insects had difficulty moulting. Histological studies showed a distortion in the cuticle and a lack in fibrous appearance. It is now known that the insecticide works by inhibiting the final polymerisation step in chitin synthesis.

Since the manufacture of the first acylurea, diflubenzuron (Dimilin®), a number of other acyl ureas have been synthesised with considerably enhanced activity, such as teflubenzuron, the insecticide of interest in this Chapter.

The mode of action of these insecticides results in newly laid down cuticle which lacks stiffness and strength due to the deficiency of chitin and the insect usually dies at the time of ecdysis. Death is caused either by a rupturing in the newly exposed cuticle leading to dehydration or by the insect becoming trapped in the old exuvae because the new cuticle lacks the rigidity required for the insect to moult properly. Death usually follows slowly as the insect is unable to move and cannot feed. However, during this period the insects are effectively dead as they possess no threat to any crops. This mode of action means that BPUs are slower acting than some pesticides, but this drawback is compensated for by its increased specificity and persistence (Bouaichi *et al.*, 1994b). This type of insecticide is known as an insect growth regulator (IGR) because its action is delayed dependent on the growth and development stage of the insect.

Although the symptoms of the insecticide are delayed, the insecticide itself acts very quickly. It has been shown that diflubenzuron begins to inhibit chitin synthesis in *Pieris brassica* larvae within 15 minutes of application (Deul *et al.*, 1978). The

effectiveness of each BPU varies from species to species and depends on a number of factors; feeding rate, level of metabolism and the dose given.

These insecticides are often viewed as being primarily effective as stomach poisons, with topical affects in many insects being typically ten fold less effective than oral doses (Mauchamp and Perrinou, 1987). However, other workers have shown that teflubenzuron used against *Spodoptera litoralis* may be equally effective as a stomach poison or by topical application (Clarke and Jewess, 1990b). However, this study found that as an inhibitor of chitin synthesis, teflubenzuron was ten fold more effective when applied as a topical dose. The effects of these insecticides are not cumulative, if the insects are given a small dose over a short period of time the disturbance caused to the cuticle will be minimal (Retnakaran *et al.*, 1989).

Dimilin, the most widely investigated BPU has no known detrimental effect on vertebrates and a limited impact on non-target invertebrates (Coppen and Jepson, 1996a). It is highly persistent and acts as a stomach poison although it has been reported to have significant contact activity in some insects (Clarke and Jewess, 1990a). It has been shown to have up to 2 months persistence in the Sahelian grassland. Teflubenzuron, however, has only 2 weeks persistence. Resistance to diflubenzuron can be induced in some insects (Brown *et al.*, 1978). Detailed studies have failed to reveal how these insecticides act, although there has been much speculation (Cohen, 1987). Theories for where this inhibition may occur are reviewed in Table 3-1.

Table 3-1. Theories for the mode of action of benzophenyl ureas

Proposed mode of action	Evidence for	Evidence against
Inhibition of chitin synthetase (CS)	Inhibition of CS *in vivo* (Post *et al.*, 1974; VanEck, 1979). *In vitro* partial inhibition (Turnball and Howells, 1983). Prevention of polymerisation of chitin oligomers also proposed as possible mechanism of BPU action (Retnakaran and Hackman, 1985)	Not effective against fungi. Many workers have found no inhibition of CS in cell free preparations (Mayer *et al.*, 1981; Cohen and Casida, 1980, Mitisui *et al.*, 1981). May involve formation of active metabolite.
Induction of a chitinase	(Ishaaya and Casida, 1974; Yu and Terriere, 1975)	Ultrastructural studies would suggest that this is not the mode of action.
Effects on epidermal DNA synthesis	Inhibition in DNA synthesis in epidermis (DeLoach *et al.*, 1981)	Does not explain why effects are seen so rapidly (Reynolds, 1987).
Inhibition nucleoside transport	(Klitchka *et al.*, 1987)	Effects on nucleoside not reversible, effects of BPUs on insects are reversible.
Blockage of transport of pre-cursors/ spatial organisation of chitin.	Inhibition of the transport of UDP-NAG across membranes (Mitisui *et al.*, 1981). Could lead to feedback inhibition of chitin synthesis (Binnington and Retnakaran, 1991).	BPUs do not inhibit pathways known to be necessary for production of dolichol phosphate carriers (Grosscurt and Jongsma, 1987)
Inhibition of protease which cleaves zymogen of chitin synthetase and activates the enzyme.	Inhibition of chymotrypsin by BPU (Mitlin *et al.*, 1977; Leighton *et al.*, 1981)	No proof that CS exists as a zymogen or that this protease is responsible for its cleavage.
Interference with hormone levels	Reduced ecdysone levels in *Tenebrio* pupae to which BPU applied (Soltani *et al.*, 1984; Soltani *et al.*, 1987)	Normal cuticle not secreted if ecdysteroids added (Soltani *et al.*, 1987). Effects seen very rapidly.

3.1.4 BPUs and locusts

There have been a number of studies on the effects of oral doses of BPUs on the desert locust. One of the earliest of these studies was carried out by Ker (1977). He found that the cuticle of diflubenzuron treated locust was prone to fracture in characteristic ways. Tears in the pharate cuticle meant that the insect was unable to ecdyse successfully, or even to move. Ker (1978) also showed that *Schistocerca gregaria* cuticle treated with diflubenzuron lacked the distinct layers present in normal locust cuticle. However, stable cuticle was deposited in the prealar arm, hind tibia and pharate mandibles. Ker proposed that in these regions protein cross-linking forms a more important part in cuticle structure. There were no reported signs of injury in fifth instars fed with diflubenzuron until the next moult.

The acyl urea of interest in this study is teflubenzuron (Nomolt™). It has been found to be the most effective of a number of acyl ureas tested against *Schistocerca gregaria*, giving the lowest LD_{50} and LT_{50} values (Coppen and Jepson, 1996a).

It has also been observed that before the moult teflubenzuron treated *Schistocerca gregaria* eat less and are less mobile (Bouaichi *et al.*, 1994b; Coppen and Jepson, 1996a). Other researchers have found that the decrease in the amount of food consumed by soybean loopers is proportional to the amount of diflubenzuron applied to the insect (Reed and Bass, 1979). At lower doses the locusts may survive to the next moult but die subsequently due to starvation (the insect fails to feed due to malformations in the mandibles) or increased water loss (due to a thinning of the cuticle). In *Schistocerca gregaria* these effects are seen more often with less potent acylureas like diflubenzuron and hexafluron.

Other sublethal effects include weakened abdominal cuticle which prevents females penetrating the soil with their abdomens and thus prevents egg laying (Grosscurt and Jongsma, 1987).

The timing of oral applications of teflubenzuron is also crucial. Coppen and Jepson (1996b) found that the nearer the application was to the next moult the more effective the dose was in second instar *Schistocerca gregaria*. It was proposed that locust pharate cuticle is predominantly laid down late in the instar. This pattern of increased cuticle synthesis before ecdysis has also been reported in fifth instar *Schistocerca gregaria* (Ker, 1977). The half life of teflubenzuron in *Schistocerca gregaria* is 24 hours.

Further effects of teflubenzuron applied to adult *Locusta migratoria* are a reduction in the number of eggs per ootheca and significant decrease in the viability of those which are laid (Onyeocha and Fuzeaubraesch, 1990). Diflubenzuron also effects the chitin and protein content of the peritrophic membrane in *Locusta migratoria* (Clarke and Temple 1977).

Successful field trials of BPUs against a number of grasshopper and locust pests have been described. Almost total control of *S. gregaria* has been reported with teflubenzuron doses between 26-35 grams of active ingredient per hectare (g.a.i. ha^{-1}) (Lecoq *et al.*, 1988). Bouaichi *et al.* (1994b) also reported significant control of *S. gregaria* in Morocco with application rates of 60g.a.i. ha^{-1} and found it to be more effective than malathion, although it was slower acting. Approximately a 90% reduction was reported in grasshopper populations in the Sahel following applications of teflubenzuron at 50-75 g.a.i. ha^{-1} (Launois *et al.*, 1988). Other workers have reported satisfactory control of younger grasshopper instars at very low teflubenzuron doses (2.3-5.3g.a.i./ha) (Krokene, 1991).

3.1.5 Temperature and insecticides

Temperature can have a great affect on the action of chemical insecticides, generally increasing temperatures leads to an increase in insecticidal activities (Ruigt, 1985). In *Manduca sexta* an increase in temperature leads to an increase in the mortality and sub-lethal effects of larvae treated with BPUs (Chandler *et al.*, 1991). This study investigated some of the possible mechanisms of this increased efficiency and found it

was not due to altered uptake of the insecticide at higher temperatures. It was proposed that differences were due to some change in the insect, either differences in the cuticle at higher temperatures making the insect more susceptible or due to some other factor affecting insect fitness.

Another study comparing the effects of temperature on the toxicity of two BPUs to a range of insects have found that the relationship between temperature and mortality depends on both the insect and the insecticide (Grosscurt and Wixley, 1991). They found that in some insects teflubenzuron had a significant decrease in activity at higher temperatures.

The temperature to which an insecticide is exposed in the field can also lead to significantly reduced stability. Work investigating the stability of two insecticides against *S. gregaria* found that at 5°C permethrin retained toxicity for between 7 and 14 days whereas at 15°C the insecticide only remained stable for 3 - 7 days (Siddiqui, 1979). However, another insecticide, fenitrothion, retained toxicity at all the temperatures investigated for at least 21 days. These differences in stability were proposed to be due to differences in vapour pressure.

3.1.6 Temperature and entomopathogens

Temperature not only affects the action of chemical insecticides but also has a serious effect on the ability of pathogens to infect their hosts, particularly in cold blooded animals, like insects, whose body temperatures can vary enormously. For example, when *Heliothis zea* are infected by *Nomuraea rileyii* the mortality rate is quite high at 20 or 25°C, however if they are kept at 15 or 30 °C mortality is significantly reduced (Mohamed *et al.*, 1977).

As was discussed in Chapter 2, these problems are often thought to be related to the reduced germination and appressorial formation rate found at sub-optimal temperatures in a number of entomopathogenic fungi (Galaini-Wraight *et al.*, 1992; Walstad *et al.*, 1970). These studies often give no consideration of the potential effects of temperature

once the fungus has entered the haemocoel. Indeed, some papers have even suggested that the fungus is 'safe' from environmental pressures once it is inside the insect.

However, Carruthers *et al.* (1991) found clearwinged grasshoppers thermo-regulate their body temperatures at about 40°C. This temperature allows highly accelerated maturation and has been found to correlate to optimal metabolic rates in a number of rangeland grasshoppers. These high temperatures also led to a severely reduced control rate with *Entomophaga grylii*, which has a thermal survival limit of 35°C. Insects which were not given the opportunity to thermo-regulate did not reduce the infection.

In the above example, there was found to be no difference in the behaviour of infected and non-infected individuals. However, even more severe problems can occur when infected individuals display what is known as behavioural fever. This is when an insect will deliberately bask to raise its temperature above normal and thus kill off an invading pathogen. This type of behaviour has been described in grasshoppers infected with *Beauveria bassiana* (Inglis *et al.*, 1996)

Not only does temperature affect the disease process in entomopathogenic fungi, it also affects the number and viability of conidia subsequently produced from cadavers (McDonald and Nolan,1995).

It is difficult to assess the effects of environmental factors on mycoinsecticides in the field due to the complexity of changes (e.g. changes in temperature can often be correlated to changes in humidity or UV radiation). For this reason it has been suggested that it is simplest to study the effects of environmental variables in the laboratory, where all other factors can be kept constant.

3.1.7 Overcoming temperature constraints in *Metarhizium spp.*

One of the suggested methods of overcoming the problems of the affects of temperature on *M. anisopliae* is the use of combined formulations of isolates with different temperature optima, for example, the combination of isolate 108 with a growth optimum of 20°C and isolate 275 with an optimum of 24°C could lead to a broader temperature spectrum (Demian and Solomon, 1986). However this kind of approach is not currently being investigated for the use of *M. flavoviride* against locust as the temperature optimum required is so high.

With *M. flavoviride* the method of formulation of the spores has a very great effect on their ability to withstand temperature extremes. It has been shown that drying *M. flavoviride* spores prior to use can lead to a significant increase in their ability to withstand high temperatures (McClatchie *et al.*, 1994)

Synergism is another way in which the ability of *M. flavoviride* IMI 330189 to overcome temperature constraints might be improved. As has been discussed previously, many insecticides become more active at higher temperatures. If this is true for the use of teflubenzuron against *S. gregaria* it may be that the increased efficiency of the insecticide at higher temperatures may help the fungus to penetrate more easily.

Synergistic interactions between benzoylphenyl ureas and *Metarhizium spp.* have been described for both *Manduca sexta* (Hassan and Charnley, 1983) and *S. gregaria* (Joshi, 1993). It has been shown in *Manduca sexta* combined formulations of diflubenzuron and *M. anisopliae* result in faster penetration by the hyphae (Hassan and Charnley, 1989). Cuticle which has been affected by BPUs is often found to be very disturbed, with a granular appearance and a lack of any lamellae. This change in cuticle structure probably means that there is less physical resistance to the penetrant hyphae and the lack of chitin may result in a more easily enzyme degradable cuticle.

A number of workers have also shown that earlier insect instars are typically more susceptible to entomopathogenic fungi than later ones, this is thought to be due to the

increase in cuticle thickness in successive instars (Schaerffenberg, 1957; Getzin, 1961 and David, 1967). If teflubenzuron is able to increase the penetrability of the locust cuticle it may have a similar effect in improving the effectiveness of *M. flavoviride* against later instars.

Another possibility may be to improve the temperature tolerance of *M. flavoviride* by inducing heat shock proteins before application, this possibility is discussed in Chapter 4.

3.1.8 Aims

The aims of the work in this Chapter were to investigate the effects of temperature on LD_{50} and LT_{50} values for *M. flavoviride* or teflubenzuron against third instar *S. gregaria*. Secondly, to look at the potential of combined doses of these two insecticides to improve the control of *S. gregaria* at higher temperatures. Finally, the effects of these two insecticides on fifth instar *S. gregaria* were also investigated.

3.2 Methods

3.2.1 *Schistocerca gregaria* culture

Locust were kept at 30°C on a 16h light:8 h dark cycle. They were fed daily with trisulfa (Appendix 2) treated bran supplemented with fresh wheat seedlings and given a supply of trisulfa treated water. Trisulfa was given to control infections with *Malamoeba locustae*.

3.2.2 Preparation of teflubenzuron

Technical grade teflubenzuron (Nomolt™; purity 100%M/M) was a gift from the Shell Research Limited, Sittingbourne, England.

It was either formulated either in ondina el oil or in acetone (in order to achieve higher concentrations). In order to dissolve teflubenzuron in oil it was necessary to heat the formulation to 50°C with continuous stirring for up to 2 hours. This formulation was allowed to cool before being mixed with spores or used to inoculate an insect. Fresh formulations of teflubenzuron in oil were made for each bioassay in order to minimize the risk of heat damaging the insecticide. In acetone the teflubenzuron dissolved easily and there was no requirement to heat the solution.

3.2.3 Antibiotic disc test

A conidial suspension of 3×10^6 conidia ml^{-1} was prepared in 0.04% Tween 80 as described in Chapter 2. 200µl of this suspension was spread on to a quarter strength SDA plate and left to dry in a Class II cabinet for 30 minutes. Sterile filter paper discs (4mm diameter) were then dipped into various concentrations of teflubenzuron dissolved in acetone and allowed to drip dry. One of these discs was placed in the centre of each plate and the plates were incubated at 27°C for 48 hours. Clearing zones around the disc were measured after this time.

3.2.4 Preparation of M. flavoviride

M. flavoviride IMI 330189 was prepared in ondina el oil as described in the Methods in Chapter 2, section 2.2.2.1.1.

3.2.5 Inoculation of *Schistocerca gregaria*

Third instar *S. gregaria* used were between 0 and 48 hours after ecdysis, fifth instar insects were between 0 and 24 hours after ecdysis. Locusts were inoculated either on the ventral surface of the thorax (for third instars) or under the pronotum (for fifth instars or adults) using a Burkhart automated inoculator. Third instar locusts were either inoculated with 1µl of formulation in acetone or 0.3µl of formulation in oil (as 1µl of oil was found to cause very high mortality even in control insects). Fifth instar insects and adults were inoculated with 1 µl of oil or acetone unless otherwise stated.

These insects were then placed in to individual containers and maintained at 28°C (unless otherwise stated) at 30% relative humidity on a 16h light:8 h dark cycle. The locusts were fed daily and their mortality recorded. Cadavers were surface sterilised in 70% ethanol and then placed on filter paper moistened with sterile distilled water in Parafilm sealed Petri dishes. These were incubated at 27°C for approximately 4-5 days to check for fungal growth and sporulation.

3.2.6 Data analysis

All data was analysed using probit analysis on Genstat, this makes it possible to linearise the data so that regression analysis can be used. For calculations LD_{50} and LT_{50} values a binomial distribution was assumed. Synergy was analysed in a similar way using assuming a binomial distribution. This programme analyses the significance of each factor in affecting mortality by analysing the pattern of mortality caused by each factor and looking for deviations from the pattern when both insecticides were applied together. These effects could be caused by synergy or antagonism. Listings of the programmes used to assess LD_{50}, LT_{50} and to test for any synergy are given in Appendix 8.

3.3 Results

3.3.1 Antibiotic disc test

An initial study on the effects of teflubenzuron on the growth of *M. flavoviride* used filter paper discs impregnated with the insecticide as described in the methods in section 3.2.3. These were placed onto ¼SDA plates which had been inoculated with *M. flavoviride*. There were no detectable zones of inhibition after 48 hours at any of the concentrations of teflubenzuron tested (up to 10μg μl^{-1}).

Table 3-2. The affect of teflubenzuron concentration on zones of inhibition in *M. flavoviride* antibiotic disc test.

Concentration of teflubenzuron (μg μl^{-1})	Zone of inhibition (mm)
0.0	0
0.1	0
1.0	0
10.0	0

Data represents the mean of 10 replicates.

3.3.2 The effects of temperature on LD_{50} and LT_{50} values caused by infection of third instar locust with *Metarhizium flavoviride*

The aims of these bioassays were to establish the interaction of temperature with *M. flavoviride*, teflubenzuron or both applied to third instar *S. gregaria*. These affects were measured in terms of LD_{50} values (median dose required to kill 50% of locusts in a given time) and LT_{50} values (median time required to kill 50% of locusts for a given dose). The significance of any interactions between *M. flavoviride* and teflubenzuron was assessed using ANOVA.

Initially temperatures of 30, 35, 40 and 45°C were investigated, but it was found that at 45°C all insects died within 24 hours. It was thought that this mortality was due to desiccation, since if the insects were supplied with a water source they could survive

this temperature. However, due to the impracticalities of this set up, temperatures of 25, 30, 35 and 40°C were used in subsequent bioassays.

In addition it was found that third instar insects could not be inoculated with 1 µl of oil as used in previous experiments described by Joshi (1993) as this lead to a very high mortality rate. Reducing the quantity to 0.3 µl removed this problem.

Initial experiments were carried out to investigate the effects of temperature on *M. flavoviride* infection. Six replicate bioassays were carried out with 4 different concentrations of conidia and five insects used per treatment. The results from the analysis of these experiments are shown in Tables 3-3, 3-4 and 3-5 and in Figure 3-2.

Figure 3-1 shows the cadavers of third instar *Schistocerca gregaria* hoppers killed by *M. flavoviride*. Cadavers were incubated at 100% humidity at 27°C for up to four days after death to check for sporulation.

To calculate the LD_{50} values regression analysis was used and the results of the fitted model are shown in Table 3.3. In all cases the single line model was found to be suitable as the residual deviance was not significant. Parallel and separate line models were also evaluated, but for all the data analysed provided no significant improvement in fit (Table 3-5). Temperature had a very great effect on the 5 day LD_{50} values for *M. flavoviride* infection (Table 3-3), the lowest value was 131 conidia per insect at 30°C. At 25 and 35°C the number of conidia required to kill half the locust were approximately 16 and 60 times greater respectively than at 30°C. At the highest temperature (40°C) there were no mortalities.

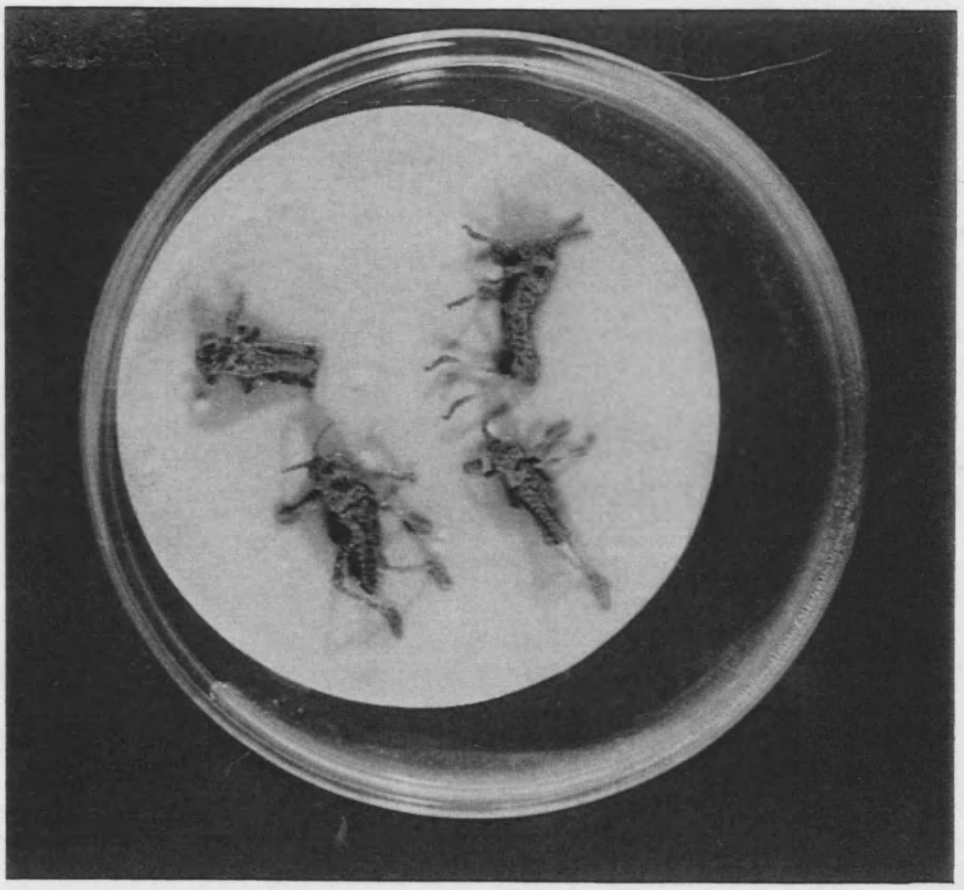
The LT_{50} was also lowest at 30°C, at about 3.4 days for insects inoculated with 3000 conidia (Table 3-3). However there were no significant differences in the LT_{50} values obtained at 25 and 35°C, although these values were both significantly higher than those at 30°C. No LT_{50} value could be calculated for experiments at 40°C as there was no mortality.

Assessments of the number of dead insects which subsequently sporulated when incubated under suitable conditions could not be made as *Aspergillus spp.* was frequently found to be the only fungus sporulating on the insect. This problem has been encountered before at Bath (Gillespie, 1995; Bye, 1993; McKemey, 1995).

3.3.3 The effects of temperature on LD_{50} and LT_{50} values caused by poisoning of third instar locust with teflubenzuron

Table 3-6 shows the 5 day LD_{50} for teflubenzuron was lowest at 40°C at 370ng per insect. This value was approximately doubled at both 30 and 35°C. At 25°C the LD_{50} dose was much higher, 69 μg per locust. The LT_{50} values appeared to be followed a very similar pattern, with 50% of mortalities at 25°C occurring within 7 days. At 30 and 35°C death occurred much faster, with 50% of insects dying within approximately 3 days. At 40°C the programme used was unable to calculate an LT_{50} value due to the mortalities on day one, therefore this value was estimated manually to be about 2 days.

Figure 3-1. Third instar *Schistocerca gregaria* cadavers sporulating with *M. flavoviride*.



This photograph shows third instar *Schistocerca gregaria* cadavers from which *M. flavoviride* is sporulating. The insects were inoculated with 3000 conidia and after death were surface sterilised and incubated on moist filter paper in a sealed Petri dish at 27 °C. Cadavers began to sporulate after 3-4 days.

Table 3-3. The effect of temperature on the five day LD_{50} and LT_{50} of third instar *Schistocerca gregaria* inoculated with *Metarhizium flavoviride*.

Temperature of Bioasssay	LD_{50} (95% c.l.) (5 days) no. of conidia per insect	LT_{50} (95% c.l.) (3000 conidia) days
25 °C	2143 (843)	4.84 (0.0776)A
30 °C	131(37)	3.39 (0.0145)B
35 °C	7314(8246)	5.10 (0.2810)A
40 °C	*	*

* Unable to obtain LD_{50} or LT_{50} values for this data. Letters indicate those values which t-tests showed to be significantly different (P=0.05).

Table 3-4. Statistical analysis of data used to generate LT_{50} values for infection of *Schistocerca gregaria* with *M. flavoviride* at different temperatures.

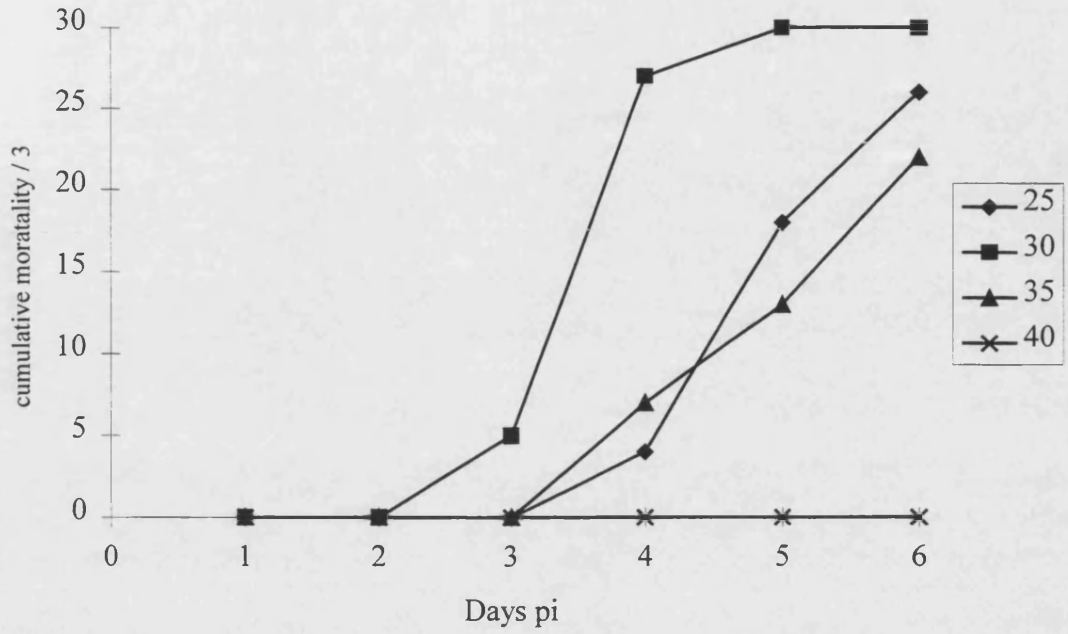
	d.f.	Deviance	Probability
Treatment	15	145.0018	<0.001
Replicates	100	44.0996	>0.995
T-test 25 - 30°C	15	2.140	0.025-0.01
T-test 25 - 35°C	15	0.024	>0.10
T-test 30 - 35°C	15	3.218	0.005-0.001

Table 3-5. Statistical analysis of data used to generate LD_{50} values for infection of *Schistocerca gregaria* with *M. flavoviride* at different temperatures

Temperature	Analysis	Deviance	d.f.	Fit to model
25°C	Single line	17.26	21	suitable model
	Parallel lines	7.06	16	no improved fit
	Separate lines	2.44	11	no improved fit
30°C	Single line	11.08	21	suitable model
	Parallel lines	8.70	16	no improved fit
	Separate lines	7.88	11	no improved fit
35°C	Single line	19.45	21	suitable model
	Parallel lines	12.50	16	no improved fit
	Separate lines	11.58	11	no improved fit
40°C	Single line	N/A	N/A	N/A
	Parallel lines	N/A	N/A	N/A
	Separate lines	N/A	N/A	N/A

'No improved fit' indicates that this model did not prove to be a significant improvement on the single line model at P=0.05, N/A indicates that statistical analysis was not possible due to the nature of the data.

Figure 3-2. Effect of temperature on the mortalities of third instar *Schistocerca gregaria* inoculated with 3000 *Metarhizium flavoviride* IMI 330189 conidia



Data points represent the number of locust mortalities out of 30 on each day post inoculation.

Figure 3-3. Third instar *Schistocerca gregaria* cadaver killed by teflubenzuron poisoning

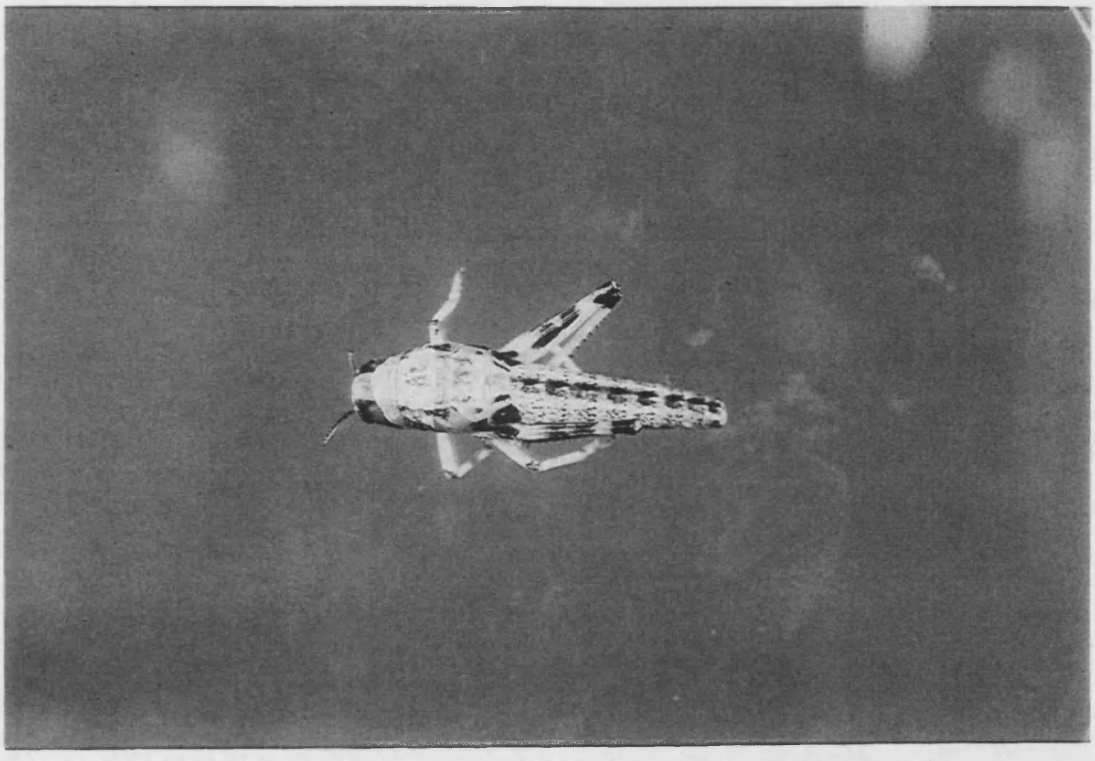


Table 3-6. The effect of temperature on the five day LD_{50} of third instar *Schistocerca gregaria* inoculated with teflubenzuron.

Temperature of Bioassay	LD_{50} (95% c.l.) (5 days) μg teflubenzuron per insect	LT_{50} (95% c.l.) (10μg teflubenzuron) days
25°C	68.6(96.5)	7.714 (1.165)
30°C	0.596(0.189)	3.360 (0.494)
35°C	0.659(0.183)	3.089 (0.247)
40°C	0.370(0.082)	1.8 *

* program unable to calculate LT_{50} value for this experiment due to mortalities on day 1 post inoculation, therefore this value was derived manually.

Figure 3-4. Effect of temperature on the mortalities of third instar *Schistocerca gregaria* inoculated with 10 μg teflubenzuron insecticide.

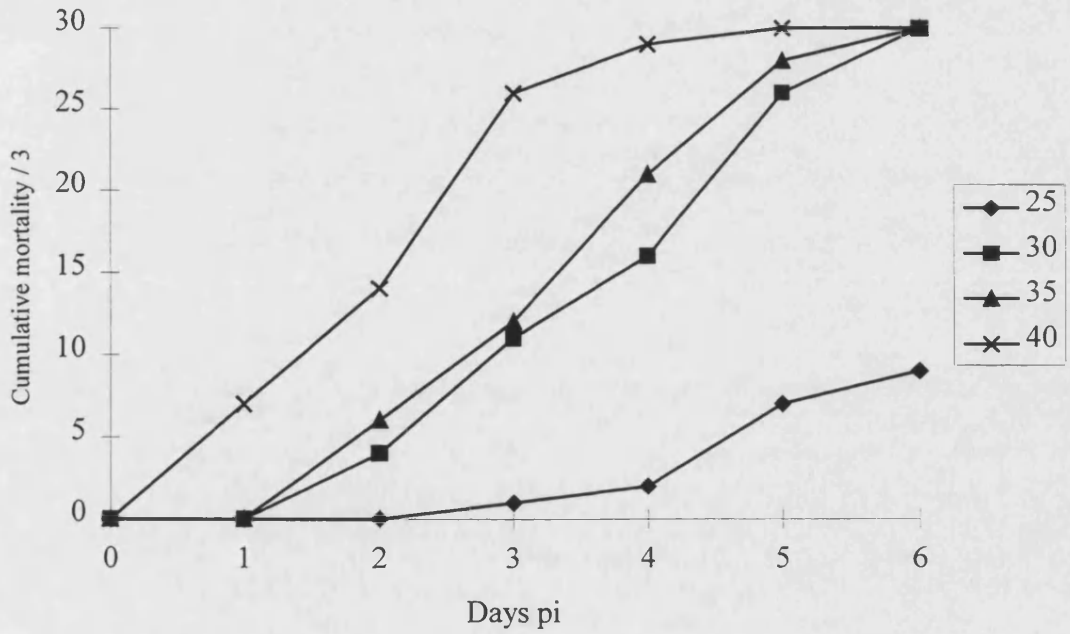


Table 3-7. Statistical analysis of data used to generate LD_{50} values for teflubenzuron against third instar *Schistocerca gregaria* at different temperatures

Temperature	Analysis	Deviance	d.f.	Probability
25°C	Single line	16.28	21	suitable model
	Parallel lines	15.28	16	no improved fit
	Separate lines	15.67	11	no improved fit
30°C	Single line	28.50	21	suitable model
	Parallel lines	33.69	16	no improved fit
	Separate lines	29.63	11	no improved fit
35°C	Single line	23.13	21	suitable model
	Parallel lines	14.66	16	no improved fit
	Separate lines	6.73	11	no improved fit
40°C	Single line	13.10	21	suitable model
	Parallel lines	5.986	16	no improved fit
	Separate lines	0.1725	11	no improved fit

'No improved fit' indicates that this model did not prove to be a significant improvement on the single line model at P=0.05, N/A indicates that statistical analysis was not possible due to the nature of the data.

Table 3-8. Statistical analysis of data used to generate LT_{50} values for inoculation of *Schistocerca gregaria* with teflubenzuron at different temperatures.

	d.f.	Deviance	Probability
Treatment	15	112.5900	<0.001
Replicates	100	71.2270	0.99 - 0.98
T-test 25 - 30°C	15	1.112	>0.1
T-test 25 - 35°C	15	0.816	>0.1
T-test 25 - 40°C	15	1.293	>0.1
T-test 30 - 35°C	15	0.374	>0.1
T-test 30 - 40°C	15	2.940	0.01 - 0.005
T-test 35 - 40°C	15	0.476	>0.1

3.3.4 The effects of temperature on mortalities caused by combined applications of *Metarhzium flavoviride* and teflubenzuron.

For bioassays using combined formulations of *M. flavoviride* and teflubenzuron the experiments were reduced to three temperatures; 25, 30 and 35°C. 40°C was not investigated because *M. flavoviride* was unable to cause infection at this temperature and because there were limited numbers of experimental animals available.

The results from this experiment are shown in Figure 3-5. Probit analysis using the Genstat programme described in Appendix 8 was used to analysed the significance of each factor in affecting mortality. This programme analyses the pattern of mortality caused by each factor and looks for deviations in the pattern when both insecticides are applied together. These effects could be caused by synergy or antagonism.

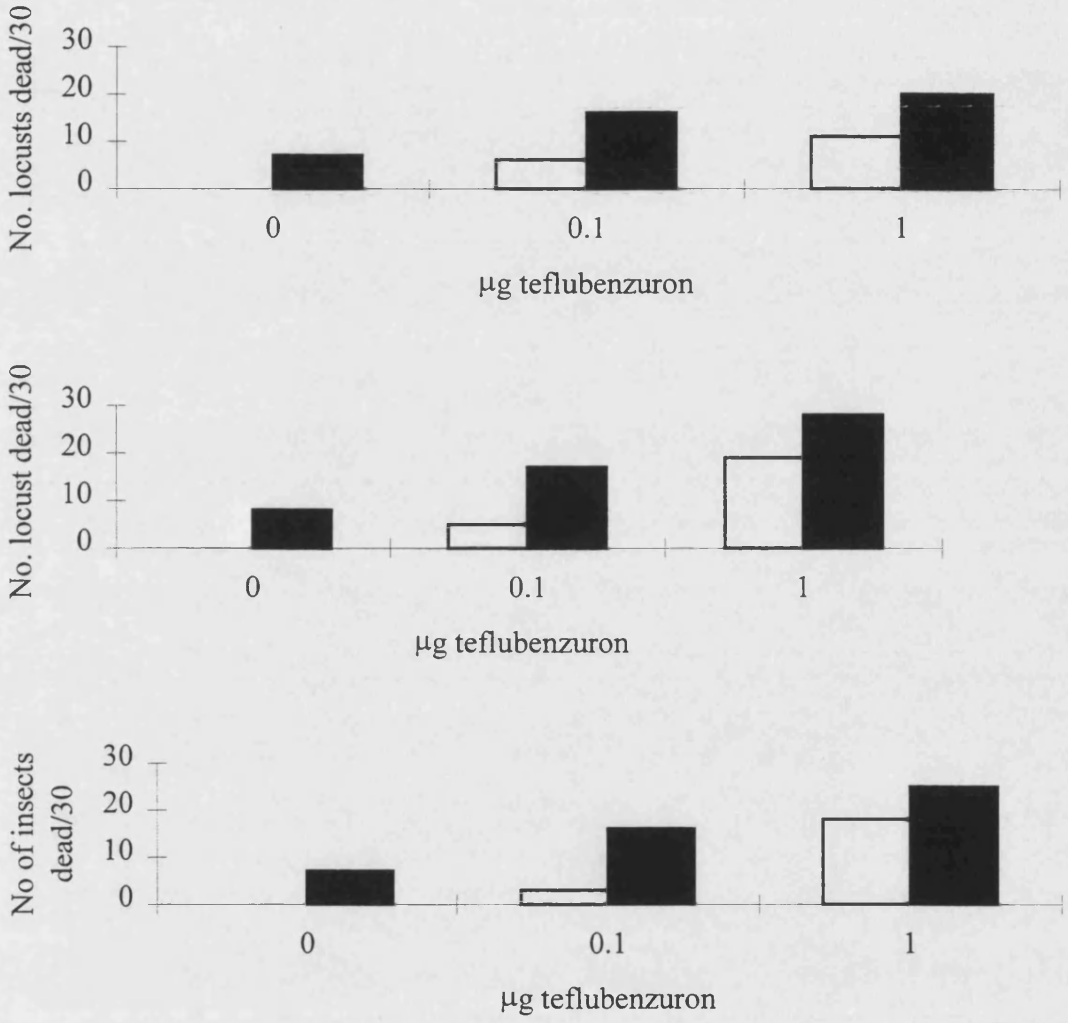
The results showed that temperature had a significant effect on mortality, as did the fungi and the teflubenzuron. However, the combined effects of *M. flavoviride* and teflubenzuron were found to significantly interact only at 35°C. From Figure 3-5 this would appear to be a synergistic interaction as there is a substantial increase in mortality in locusts which have been inoculated with 0.1 μg of teflubenzuron if *M. anisopliae* is added at the same time. In bioassays carried out at other temperatures there was no significant interaction between *M. flavoviride* and teflubenzuron.

Table 3-9. Probabilities that components of synergy experiment have a significant effect on locust mortality.

		d.f.	Deviance	F value	Effect on mortality
25°C	Replicate	5	3.7505	0.936	-
	M. flavoviride	1	20.261	25.273	++
	TFB	2	61.804	38.546	++
	Interaction *M. flavoviride* and TFB	2	3.138	1.954	-
	Residual	25	20.043		
30°C	Replicate	5	4.630	0.861	-
	M. flavoviride	1	18.710	17.388	++
	Teflubenzuron	2	64.827	30.125	++
	Interaction *M. flavoviride* and TFB	2	2.741	1.274	-
	Residual	25	26.902		
35°C	Replicate	5	3.292	1.056	-
	M flavoviride	1	15.434	24.749	++
	Teflubenzuron	2	58.090	46.576	++
	Interaction *M. flavoviride* and TFB	2	4.603	3.692	+
	Residual	25	15.590		

Data was analysed by the using ANOVA with the GENSTAT programme for synergy listed in Appendix 8. - indicates that there is no significant effect on mortality, + indicates that there is a significant effect on the mortality of locust at the 0.05 level and ++ indicates a significant effect at the 0.001 level.

Figure 3-5. Effect of temperature on the mortalities of third instar *Schistocerca gregaria* inoculated with teflubenzuron insecticide, with or without *Metarhzium flavoviride* IMI 330189 infection after 6 days.



Unshaded bars represent mortality with teflubenzuron alone, shaded bars represent mortality in insects infected teflubenzuron and 300 *M. flavoviride* IMI 330189 conidia

3.3.5 The effects of *Metarhzium flavoviride* and teflubenzuron on fifth instar *Schistocerca gregaria.*

Further work was carried out to look at the effects of teflubenzuron and *M. flavoviride* on fifth instar *S. gregaria*. Temperature was not used as a variable in this experiment so the experiments were carried out at the standard bioassay temperature of 27°C. Figure 3-6 shows the mortality over 6 days for fifth instar *Schistocerca gregaria* inoculated with 30,000 spores. The LD_{50} after five days was found to be 1388 (±239) conidia/insect and the LT_{50} for insects infected with 30,000 conidia was found to be 4.04(±0.298) days.

Experiments using topical doses of teflubenzuron on fifth instar locusts showed that there was no significant mortality at any of the doses used (up to 10 $\mu g\ \mu l^{-1}$, results not shown). Therefore no LT_{50} or LD_{50} data could be generated from these experiments.

Due to the lack of mortality in fifth instar *Schistocerca gregaria* topically inoculated with teflubenzuron, preliminary investigations were also carried out to look at the possibility of using this IPM system as an oral dose. Locusts were starved for 24 hours prior to the experiment and then given a 1cm strip of wheat seedling onto which 1µl of teflubenzuron or *M. flavoviride* in oil formulation had been placed. Controls were fed wheat seedlings with 1µl of ondina el oil. It was found that an oral dose of $1\mu g\ \mu l^{-1}$ teflubenzuron in ondina el oil was sufficient to kill all the insects at the next moult. Preliminary investigations also showed that oral doses of *M. flavoviride* were able to kill fifth instar *Schistocerca gregaria* although there was a substantial increase in the time taken to kill (time to death was increased by about 2 days for a dose of 3×10^5 conidia $insect^{-1}$). Interestingly insects treated this way turned a very bright pink colour before death (Figure 3-7). This pink colouration was observed in all mycosed insects prior to death but was particularly obvious in these insects fed *M. flavoviride* conidia.

Figure 3-6. Cumulative mortality of fifth instar *Schistocerca gregaria* topically infected with 30,000 conidia

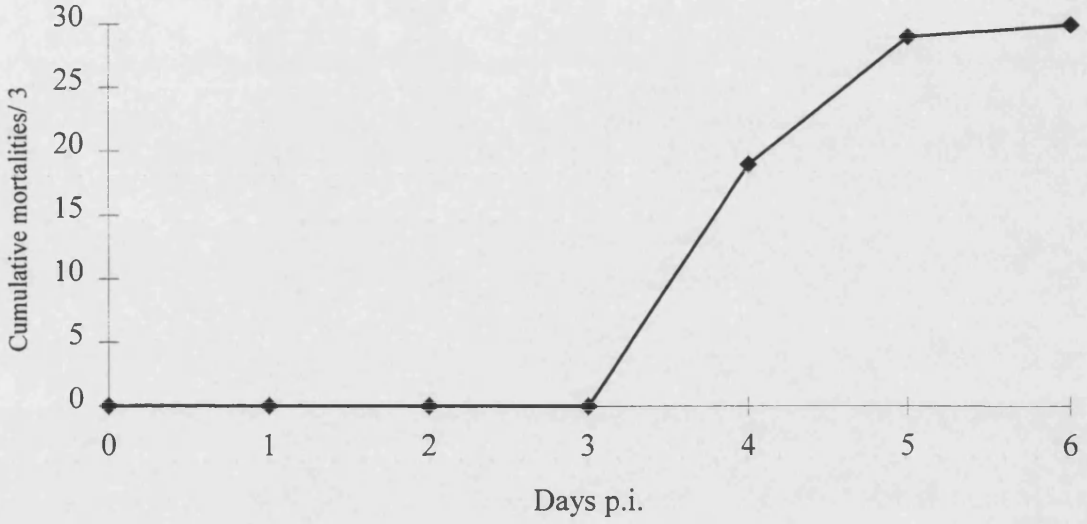
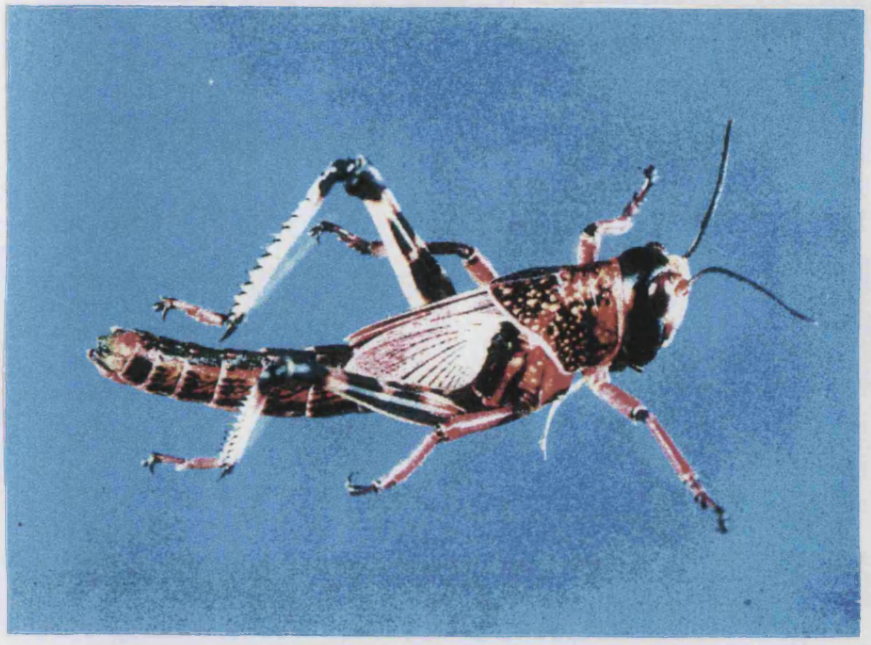


Table 3-10. Statistical analysis of the data used to generate LD_{50} values for *Metarhizium flavoviride* against fifth instar *Schistocerca gregaria* at 27°C.

Analysis	Deviance	d.f.	Probability
Single line	16.28	21	suitable model
Parallel lines	15.28	16	no improved fit
Separate lines	15.67	11	no improved fit

Figure 3-7. Cadaver of fifth instar *Schistocerca gregaria* fed 30,000 *Metarhzium flavoviride* conidia.



Discussion

3.4.1 Effects of teflubenzuron on *Metarhizium flavoviride* growth.

Joshi (1993) showed that teflubenzuron did not affect the survival of *M. flavoviride* conidia if both were stored together in oil formulation, even over long periods of time. Initial experiments in this study on the effects of teflubenzuron on *M. flavoviride* growth extended this work. Teflubenzuron was found not to inhibit the growth of the conidia *in vitro* at any of the concentrations investigated. Hassan and Charnley (1983) used the same method to demonstrate that diflubenzuron, another benzoyl phenyl urea, had no effect on the growth and germination of *M. anisopliae*. However, diflubenzuron has been found affect other entomopathogens, slightly inhibiting the growth of *Verticillium lecanii* (Hall, 1981) and enhancing the growth of *Nomuraea rileyii* (Gardener *et al.*, 1979).

3.4.2 The effects of temperature on *Metarhzium flavoviride* infection in third instar hoppers

An investigation on the effects of temperature on *M. flavoviride* infection of third instar *S. gregaria* showed that the optimum 5 day LD_{50} value of 131 conidia/insect was obtained at 30°C. The LD_{50} value calculated by Joshi (1993) carrying out similar bioassays on third instars dipped in water formulated *M. flavoviride* at 28°C in water suspension was 4.3×10^{7} conidia ml^{-1}. Of the temperatures investigated during this experiment, 30°C most closely matched *M. flavovirides*' optimum growth temperature on quarter strength SDA and was the temperature shown to give rise to maximum germination and appressorial formation *in vivo* in Chapter 2.

At 25°C and 35°C there were increases in the LD_{50} values. At 25°C the LD_{50} value obtained was 2143 conidia $insect^{-1}$ and at 35°C it was 7314 conidia $insect^{-1}$. Interestingly, these LD_{50} values also correlate well with the results described in Chapter 2; where growth, germination and appressorial formation were found to be highest at 30 °C, slightly lower at 25°C and much reduced at 35°C.

At 40°C there was no mortality at all in third instar *Schistocerca gregaria* infected with *M. flavoviride*, presumably because the fungus was unable to germinate and therefore infect the insect at this temperature. Previous experiments in Chapter 2 have demonstrated that *M. flavoviride* is unable to grow on quarter strength SDA at this temperature, so it is perhaps not surprising that it cannot infect its host at this temperature either.

The LT_{50} values also followed the same pattern, with the quickest kill obtained at 30°C and slower speeds of kill obtained at 25 and 35°C. Previous work showed that *M. anisopliae* isolates had LT_{50} values which were inversely proportional to temperature (Moorhouse *et al.*, 1994). However the temperatures used in this investigation were comparatively low (10-20°C) and this result is therefore not unexpected. This work also showed that the comparative virulence of each isolate was dependent on the temperature. Other researchers have shown that *M. anisopliae* infection at low (Roth *et al.*, 1995) and high temperatures (Soares *et al.*, 1983) can also be inhibited. Similar experiments by Mohamed *et al.* (1977) showed that mortality of *Heliothis zea* infected with *Nomuraea rileyi* was high when the insects were reared at 20 or 25°C, but was much reduced at 15 and 30°C. Reduced virulence in all these experiments probably resulted from an inhibition of germination and eventually spore or germling death.

It is important to note that although the environmental temperature in these experiments is known, the temperature in the micro-environment on various different parts of the locust could be very different. For example, the temperature of adult locust in flight can be up to 7°C higher than the ambient temperature and warm weather is known to stimulate locusts to fly more (Foster and Robertson, 1992; Xu and Robertson, 1994). This affect will make infection of adult locust with *M. flavoviride* even less likely at high temperatures.

3.4.2 The effects of temperature on LD_{50} and LT_{50} values caused by teflubenzuron poisoning in third instar *Schistocerca gregaria* hoppers.

Bioassays showed that the LD_{50} and LT_{50} values due to poisoning with teflubenzuron were inversely proportional to temperature for the most part. The LD_{50} of teflubenzuron decreased dramatically with increasing temperature, however there was no significant differences in the mortalities caused at 30 and 35°C. At 25°C the dose of teflubenzuron required to kill 50% of the insects in 5 days was impracticably high (68.6μg $insect^{-1}$). At 30 and 35°C the dose required was reduced 100 fold and at 40°C was half this again.

The results found in this study correlated well with those described by Coppen and Jepson (1996a), they found an LD_{50} value of 0.71 μg of teflubenzuron per insect in second instar *Schistocerca gregaria* kept under a variable temperature regime(Coppen and Jepson, 1996a).

The programme used to generate LT_{50} values failed to produce a value for insects at 40° C because of the mortality on day 1 post inoculation. However, from the data it is clear that insects died much faster when kept at 40°C (Figure 3-4). The LT_{50} values for third instar insects poisoned with teflubenzuron followed the same pattern as the LD_{50} values.

Differences in the speed of kill caused by teflubenzuron at different temperatures can be explained by the change in development rates of *S. gregaria*. It is common in grasshoppers to find the insect basks in order to increase its body temperature and this in turn has been found to lead to a much increased development rate (Carruthers *et al.*, 1991). The development of the third instar *S. gregaria* nymphs used in this experiment was also found to be greatly influenced by the temperature at which the experiment was carried out and moulting occurred earlier in insects maintained at higher temperatures (insects kept at 40°C typically moulted about 2 days earlier than those maintained at 25° C). As the effects of teflubenzuron poisoning are primarily seen at the moult, it is clear that insects moulting earlier are also likely to die earlier.

The reasons for the increased efficiency of teflubenzuron at higher temperatures are not known. Chandler *et al.* (1991) proposed that the increased susceptibility of *Manduca sexta* to benzoyl phenyl ureas at higher temperatures was due to some change in the cuticle or an altered fitness of the insect. However, there is an alternative explanation. The half life of oral doses of teflubenzuron in *S. gregaria* is known to be about 24 hours (Coppen and Jepson, 1996b). If we presume that the half life does not change with temperature then differences in mortality at different temperatures can be explained by the advanced development rate of insects maintained at higher temperatures. As mentioned previously, third instar *S. gregaria* kept at 40°C were found to moult about 2 days faster than those kept at 25°C. There have already been a number of reports that show that the majority of new cuticle is laid down towards the end of the inter-moult period in *S. gregaria* (Coppen and Jepson, 1996b; Ker, 1977). This would mean that at 40°C there would be a more rapid deposition of cuticle and therefore that the teflubenzuron has more chitin to influence at an earlier stage. Insects kept at lower temperatures mature more slowly and the insecticide may well have been substantially broken down by the time the insect reaches the same developmental stage, so that there is less teflubenzuron available to influence the same rate of chitin deposition.

The differences in the LD_{50} and LT_{50} values found in this study and those found by Joshi (1993) may be explained by a number of factors. Firstly it was evident from applications of oil that in this study 1µl of oil caused a high degree of mortality amongst third instar locusts, however, the same results were not found by Joshi, who found relatively little mortality amongst inoculated third instar with this volume of oil. These differences could be explained by two things. Firstly, the method of carrying out bioassays with locust at Bath leads to certain biases as locusts are not chosen on an entirely random basis. Locusts are caught by hand from a large cage containing many individuals and this means that larger and slower moving insects are caught more easily. This in turn means that these locusts are not able to breed and therefore an unnatural selection pressure is made on the insects. Comparison of the *S. gregaria* at Bath with

those insects kept at IIBC (who have recently outbred their population of locust with a fresh stock of *S. gregaria*) shows that the insects kept at Bath are much smaller. This selection pressure may have lead to differences in the locusts over time which could account for the differences in results. Secondly, the picture of a third instar locust in Joshi (1993) is actually a fourth instar insect and use of the wrong instar in some bioassays may have lead to further differences between his results and those found during this study.

It is also possible that the teflubenzuron lost some of its activity over time. However, Joshi (pers. comm.) also reported similar results with preliminary experiments using the teflubenzuron against fifth instar insects.

3.4.3 Combined applications of teflubenzuron and *Metarhzium flavoviride.*

The usefulness of teflubenzuron as an IPM strategy is somewhat in doubt from this study. As this insecticide is frequently quoted as being more effective as a stomach poison it is possibly not the best choice of insecticide for use in such a strategy. One problem with this insecticide is that frequently the adult stage is the one which is being controlled and as the insecticide kills primarily at the next moult it is not going to be very effective against adults, although it may achieve some control of the subsequent offspring. It will also affect post-ecdysial cuticle in the adults and may increase their susceptibility to *M. flavoviride*. Teflubenzuron may have some potential value in IPM strategies which are individually tailored by local workers. However, as there is a lot of variation in the control achieved at different instars the data is complicated to work with. In addition other work would suggest that in *S. gregaria* the optimal timing for application of teflubenzuron is 24-48 hours prior to ecdysis. Conversely, it might be expected that the best period for application of *M. flavoviride* would be immediately after a moult, when the cuticle layer is at its thinnest and there is the least chance of the inoculum being lost during ecdysis.

Synergy with these components against third instar *Schistocerca gregaria* has been described before by Joshi *et al.*, 1992. However in this study synergy occurred only at the highest temperature investigated 35°C. Joshi (1993) found that fungus alone took about 63 hours to penetrate through third instar *S. gregaria* cuticle. However, if the fungus was applied with teflubenzuron cuticular penetration was achieved ten hours earlier. No differences were observed in the germination and appressorial formation rate.

Synergism with acylureas has also been investigated with a number of other chemical insecticides such as hydrolase inhibitors and glutathione S-transferase inhibitors (Van Haeke and Degheele, 1991). These combinations of insecticides gave up to a nine fold increase in mortality to the cotton bollworm when applied jointly with diflubenzuron or teflubenzuron.

The use of deltamethrin, a pyrethroid against *Locustana pardalina* in Karoo, South Africa led to behavioural changes in the insects which increased their susceptibility to the locust fly. The insecticide increased the frequency of natural parasitism by 30% and the fly appeared to be unaffected by the insecticide (Armstrong, 1993).

It is possible that IPM strategies involving *M. flavoviride* and a chemical insecticide in the field may have other implications. If *M. flavoviride* is found to induce behavioural fever in *S. gregaria*, then joint application with a chemical insecticide which becomes more active at higher temperatures will lead to an increase in mortality. In effect *M. flavoviride* will induce the insect to behave in a way which will make it more susceptible to the chemical insecticide. Other researchers have shown that combined applications of *M. flavoviride* and cypermethrin can act synergistically against the migratory locust, inhibiting feeding and advancing mortality by 48 hours (Sanyang and Van Emden, 1996).

3.4.4 Infection of fifth instar *Schistocerca gregaria* with *Metarhzium flavoviride*

The five day LD_{50} dose for third instar *Schistocerca gregaria* infected with *M. flavoviride* in oil formulation and maintained at 30°C is 131 conidia per insect and for adult locust is 35,000 conidia per insect. The five day LD_{50} dose for fifth instar *Schistocerca gregaria* is between these two values 1388 conidia per insect. It is interesting that in this study mortality was found in orally infected fifth instar *S. gregaria*, as Dillon (1984), had previously noted that there was no infection of fifth instars via oral doses of *M. anisopliae*. He also found that spores passed very quickly through insects gut and were subsequently found to have a reduced viability. This would go some way to explaining the increased time to death observed in locusts fed doses of *M. flavoviride* compared to those which were given the same dose topically. The infection in this study was most likely caused by spores adhering to the mouth parts.

However, there are problems with oral doses of this combined formulation. *M. flavoviride* has been found to decrease feeding in adult locusts, and thus the insects would have to consume a sufficient dose initially to cause a fatal infection (McKemey, 1995).

3.4.5 Teflubenzuron and fifth instar *Schistocerca gregaria*

No control of fifth instar *Schistocerca gregaria* was obtained with teflubenzuron in this study, similar results were also reported by Joshi (pers. comm.). However, it has been found that topical doses of teflubenzuron in fifth instar *Schistocerca gregaria* do lead to a reduction of over 50% in the chitin content of the cuticle (Joshi, 1993). In general locust insecticides are more effective on the younger instars and the adult stages and less effective on the fourth and fifth instars. This is not always the case however, the reverse has been found to be true for migratory locust which have been fed doses of teflubenzuron. Obviously this has not proved to be the case for topical doses of

teflubenzuron in desert locust, which have been found to be much more effective on third instar than fifth instar insects.

4. Heat Shock Proteins in *Metarhizium flavoviride* and *Metarhizium anisopliae.*

4.1 Introduction

Results from Chapters 2 and 3 have shown that extremes of temperature cause serious problems in using *M. flavoviride* as a biological control agent. They lead to reduced germination, appressorium formation and growth as well as increasing the LD_{50} and LT_{50} of infected *S. gregaria*. There are a number of steps which could be taken in order to improve the ability of *M. flavoviride* to infect insects at higher temperatures but one particular aspect which is investigated in this Chapter is the induction of heat shock proteins.

Extreme temperature affects organisms by damaging the cell in a number of ways. Firstly, an increase in temperature leads to the denaturation of proteins by breaking down the non-covalent bonds which hold the protein in its tertiary and quarternary structures. This is often thought to be the major factor relating to cell death as there is a strong relationship between enzyme thermostability and innate thermotolerance. The nucleic acids and membranes are also disrupted at higher temperatures giving rise to excessive membrane fluidity. Mitochondria may become damaged leading to loss of respiratory control. Other changes that occur within the cell include disruption of the Golgi complex and transient damage of the nucleoli leading to the blockage of the assembly and export of ribosomes.

Fungi can limit these damages by a number of mechanisms which are discussed in detail in this introduction; the production of heat shock proteins, the build up of chemicals which increase thermostability (trehalose and polyols) and changes in lipids to alter membrane fluidity.

4.1.1 Heat Shock Proteins

Heat shock (or stress) proteins (HSPs) are produced in all organisms studied to date, from archaebacteria, through eubacteria, fungi, plants, invertebrates to vertebrates (Morimoto *et al.*, 1990). Comparisons of deduced amino acid sequences and use of antibodies has shown them to be among the most highly conserved proteins known in nature. For example, HSP70s in eukaryotes show between 50-98% homology in their amino acid sequence with some areas of the protein being more conserved than others. This conservation of structure and function throughout evolution points towards the extreme importance of heat shock proteins. These properties have also made the proteins very useful models for phylogenetic analysis between a wide range of organisms (Gupta, 1995).

4.1.2 Heat Shock Response

Heat shock proteins are induced in response to changes in temperature and also a number of other forms of stress such as starvation (Zoeger *et al.*, 1992), oxidative stress, hypothermia (Kapoor and Lewis, 1987), infection (Kao and Nevins, 1983), metabolic inhibitors (Johnston *et al.*, 1980) and chemical stress (Li and Hahn, 1978). Different stresses give rise to different responses, but one stress will often provide cross-protection against another stress (Mager and Kruijff, 1995). These differences in responses are being developed commercially as a method of monitoring environmental pollution (Mager and Kruijff, 1995).

The increased production of HSPs during heat-shock is often correlated with a number of other changes such as a dramatic reduction in the synthesis of other proteins (Schlesinger *et al.*, 1982). Once the source of stress is removed the synthesis of heat shock proteins rapidly declines. For a while there is repression of all protein synthesis and then there is a gradual increase in normal cellular protein production. The distribution of the HSPs within the cell also changes during this recovery period. The length of time it takes the cell to recover is dependent on the temperature of the heat shock; the higher the temperature the longer the recovery time (Howarth and Ougham,

1993). Heat shock proteins have also been found to be regulated according to the developmental or cell growth stage of the organism (Hahnel *et al.*, 1986; Herberts *et al.*, 1993, Patterson and Kapoor, 1995).

HSPs were first discovered in *Drosophila melanogaster* in the early 1970s, but it was not until the end of the decade that studies started to reveal how universal these proteins were (Morimoto *et al.*, 1990). We now know that there are a number of different families of HSPs that are classified according to their molecular weight and which are structurally unrelated. These are small HSPs, HSP60, HSP70, HSP90 and large molecular weight HSPs (Lindquist, 1986). Each family of proteins has a different role within the cell and some of these roles are discussed later.

4.1.3 Heat Shock Proteins as molecular chaperones

A more accurate term for an HSP is a molecular chaperone, as many are now known to have essential functions under normal cell conditions. They are implicated in all the major growth processes - cell division, DNA replication, transcription, translation and membrane function (Mager and Kruijff,1995).

For many years it was assumed that the folding of proteins within the cell occurred spontaneously, with the conformation determined only by the amino acid sequence of the protein, as has been shown *in vitro* with some isolated proteins. However, it is now known that molecular chaperones, like the HSPs, play a key role in the folding of proteins within the cell.

The definition of a chaperone is a protein that binds to and stabilises an otherwise unstable conformer of another protein and by controlled binding and release, facilitates its correct fate *in vivo*, be it folding, oligomeric assembly, transport to a particular sub-cellular compartment or its disposal by degradation (Hendrick and Hartl, 1993). HSPs assist in protein formation by blocking non-specific protein-protein interactions by binding to and temporarily stabilising the protein, thus preventing aggregation. The correct folding of the protein is then facilitated by controlled release of the protein

allowing it to attain its native state (Hartl *et al.*, 1994). The HSPs bind to hydrophobic sites that are normally buried within the protein but which become exposed when the proteins are newly synthesised or damaged. HSPs also enhance the flow of substrates through the proteolytic pathways known to be responsible for the degradation of aberrant proteins (Parsell and Lindquist, 1993). These are also believed to be the methods HSPs employ to protect the cell from some of the ill effects of stress (Craig *et al.*, 1993). Their mode of action means that they increase the yield of correctly folded protein but not the rate at which it is produced, thus distinguishing them from folding catalysts like protein sulphide isomerases which accelerate slow steps in the folding of some proteins.

As mentioned previously, heat shock in cells can be correlated to a rapid increase in the production of HSPs and a drop in the production of other proteins. This decline in protein production can be caused by a number of different mechanisms - changes in the rate of transcription of the ribosomal genes, RNA processing, mRNA stability and translation and transcriptional termination (Morimoto *et al.*, 1990). Other genes may also be induced at the same time; such as those encoding carbohydrate, lipid and protein metabolising enzymes (Howarth and Ougham, 1993). There are also increases in the quantity of polyubiquitin, which is probably involved in the proteolysis of denatured proteins. Morphological changes can also occur with heat shock, such as the change from hyphal to yeast growth forms that occurs in *Histoplasma capsulatum* and a number of other dimorphic human pathogenic fungi with an increase in growth temperature from 25°C to 37°C. There is evidence that small HSPs act as the trigger for this morphological switch by effecting actin polymerisation (Goldani *et al.*, 1994; Rahman *et al.*, 1995) .

Increases in the production of HSPs during stress can be very dramatic, for example, in *Drosophila melanogaster* there is a one thousand fold increase in the production of HSP70 during heat shock (Klemenz *et al.*, 1985). Although the response is not as

dramatic in most organisms, it still exists and in many cases is partly due to the possession of a number of different *hsp70* genes which are under different regulation (Ingola *et al.*, 1982). Yeast cells, for example, possess at least 9 copies of the *hsp70* gene, some of which are under constitutive control and others of which are regulated by heat shock. Homologues of the heat shock proteins which are not temperature induced are sometimes known as heat shock cognates, or HSCs.

4.1.4 Induction of Heat Shock Proteins at the molecular level

In eukaryotes, those genes which are induced by heat shock generally do not contain introns and always have a sequence motif contained in the 5' flanking region referred to as the heat shock element or HSE. This sequence consists of 3 to 6 inverted multimers of the nucleotide sequence NGAAN which has been shown to be the binding site for heat shock transcription factor (HSF). Heat shock factor is encoded on a single copy gene which is transcribed constitutively and is essential for viability in *Saccharomyces cerevisiae*. Other organisms have up to three copies of the gene, some of which are involved in development and others which are involved in stress. HSF consists of an N terminal DNA binding domain, followed by leucine zippers which are responsible for the trimerisation of the protein which is required for high affinity binding to DNA. At the C terminal end there is a heptad repeat thought to be responsible for sustaining the response. During non-stress conditions the protein is found in the monomeric form, with no detectable DNA binding activity. On the application of heat shock, trimers of HSF form and accumulate within the nucleus of the cell. Binding and activation of the HSE can be detected within minutes. The HSF stimulates a 10 - 20 fold increase in transcription, depending on the severity of the heat shock. On removal of the heat source the HSF dissociates into monomers and redistributes within the cell. Multiple forms of transcriptional regulation (for example, by other forms of stress) are mediated by the presence of additional non-HSE promoter elements (Morimoto *et al.*, 1990). How heat shock is detected by the cell is not yet known although it is thought that it could be through the appearance of denatured proteins (Magar and Kruijff, 1995;

Karemori *et al.*, 1994). In eukaryotes the heat shock response is thought to be primarily regulated by levels of HSP70 within the cell which is known to interact with HSF (Craig and Gross, 1991). It is thought that on heat shock all free HSP70 becomes bound to proteins and thus releases the HSF to form trimers and induce the transcription of heat-shock genes.

4.1.5 Thermotolerance and phenocopy induction

The increased production of heat shock proteins can also be correlated to thermotolerance and protection from phenocopy induction; i.e., heat shock at a mild temperature can protect an organism from higher temperatures which may be lethal or lead to abnormalities during development (Morimoto *et al.*, 1990). Although thermotolerance is thought to be related to the production of HSPs there is as yet only indirect evidence. It certainly cannot be attributed to any one HSP, indeed the correlation of HSP production and thermotolerance depends on the organism in question (Stege *et al.*, 1995). In *Saccharomyces cerevisiae* it appears that HSP104 is the major protein responsible for thermotolerance, but *Drosophila melanogaster* does not produce an HSP100 and in this organism HSP70 appears to be of primary importance (Parsell *et al.*, 1993). Thermotolerance and HSP production is also important from a ecological point of view, although as yet very little work has been done in this area. Species which are very closely related can show enormous differences in their thermotolerance and in the production of HSPs which can often be related to their environment (Coleman *et al.*, 1995).

An example of the possible role of HSPs in preventing phenocopy induction is provided by yeast, where an intermediate heat-shock protects against mutations which occur at extreme temperatures. These anti-mutagenic effects are not seen if the yeast is first exposed to cyclohexamide which prevents it from making protein. It is proposed that heat-shock leads to the induction of proteins which repair pre-mutational damage by

making other proteins resistant to denaturation (Nunes *et al.*, 1993). The same pattern of protection against phenocopy induction can also be seen in *Drosophila melanogaster*.

4.1.6 HSP70 and HSP90

The two most commonly studied HSPs are HSP70 and HSP90. Both are essential under normal cell conditions, but are expressed at higher levels during exposure to stress. The major differences in these two proteins are listed in Table 4-1.

4.1.6.1 HSP70

HSP70s have essential roles under both stress and non-stress conditions, including *de novo* protein folding, membrane translocation, the degradation of mis-folded proteins and regulatory processes. HSP70 protects against nuclear aggregation by binding and releasing hydrophobic sections of unfolded polypeptide in an ATP hydrolytic reaction cycle. The binding-release cycle of HSP70 is modulated by other proteins in the system, such as HSP40. The *hsp70* gene consists of an N-terminal ATPase domain of about 45kDa, followed by about 18kDa containing the peptide binding site and a more variable 10kDa. The ATPase domain transmits ATP-dependent conformational changes to the peptide-binding domain. The *hsp70* gene family is usually a large multi-gene family.

4.1.6.2 HSP90

HSP90 is distinguished from other chaperones in that it has a very high degree of specificity for the proteins it binds. It is involved in the activation of some kinases (Csermely *et al.*, 1994). It is also involved in maturing steroid receptors, but only binds some members of the steroid receptor family, such as the glucocorticoid and progesterone receptors. It is essential for cell viability in yeast. These proteins are present in abundance under normal cell conditions but can be further induced during stress, sporulation and stationary phase (Mager and Kruijff, 1995). Generally there are fewer family members of HSP90 than HSP70, for example there is only one gene in *Escherichia coli* and *Drosophila melanogaster* and two in *Saccharomyces cerevisiae*.

Table 4-1. A comparison of HSP70 and HSP90.

	HSP70	HSP90	Reference
Prokaryotic proteins	DnaK.	HtpG.	Parsell and Lindquist, 1993
Eukaryotic proteins	grp 78, HSC 70, BiP, Kar2, Ssa, Ssb, SSc.	HSP 82, 83, 87, grp 94.	Craig *et al.*, 1993
Monomer size	67-78 kDa.	82-96 kDa.	Parsell and Lindquist, 1993
Location	Location dependent on specific family member; cytoplasm, nucleus, ER, mitochondria, chloroplasts.	Cytoplasm, nucleus, ER. No association with specific organelles.	Parsell and Lindquist, 1993
Family size	Large multi-gene family.	Single or small multi-gene family.	Craig *et al.*, 1993
Normal functions	Molecular chaperone, protein assembly and membrane translocation; secretion, imports to organelles acts non-specifically. Dissociate some protein aggregates.	Essential for yeast viability, not essential in *E. coli*. Interact with specific steroid receptors, kinases, etc. to promote activity and escort to proper cellular compartment. Autophosphorylation activity.	Parsell and Lindquist, 1993 Jakob and Buchner, 1994.
Heat stress	Promotes growth at moderate temperatures, increases survival at higher temperatures. Expression increased up to 1000 fold.	Quantity produced proportional to temperature. Not essential for tolerance of high temperatures. Found in nucleus during heat shock. Expression increased 2 - 3 fold.	Borkovich *et al.*, 1989 Mager and Kruijff, 1995
ATP	ATPase activity. Action dependant on ATP.	ATPase activity. Not all activities dependant on ATP.	Weich *et al.*, 1992

Amino acid homology	50% amongst eukaryotes.	50% amongst eukaryotes 40% between eukaryotes and *Escherishia coli.*	Cheng *et al.* 1993

HSP90 is often found in complex with other proteins (HSP70, p60 and immunophilins) and these associations have been shown to be conserved between higher and lower eukaryotes. The role of these interactions is as yet poorly understood although it is known that HSP70 and p60 promote interactions with at least some of the target proteins (Chang and Lindquist, 1994). Indeed HSP70 has been shown to be essential in the process of forming heterocomplexes between HSP90 and steroid receptors in the high affinity biding state (Hutchinson *et al.*, 1994).

HSP90 directly induces the condensation of chromatin and it is thought that this may serve to regulate the transcriptional activity of steroid receptors (Csermely *et al.*, 1994). It also interacts with proteins responsible for the cell cytoskeleton; actin and tubulin and with HSF.

4.1.7 Other methods of protecting from stress

Other factors also play a part in stress tolerance in fungi and yeast. For example, the accumulation of trehalose in *Saccharomyces cerivisiae* matches the acquisition of a stress tolerant state and it is these sugars, not HSPs, that are associated with differences in barotolerance in this organism. In both *Saccharomyces cerivisiae* and *Schizosacchoromyces pombe* the accumulation of trehalose is induced by the same factors that induce HSPs - heat shock, nutrient limitation, heavy metals, oxidants and organic solvents (Fujii *et al.*, 1996). Physiological concentrations of up to 0.5M trehalose have been found to efficiently protect the activity of a number of enzymes *in vitro* from yeast, bovine and bacterial origins from extremes of temperature (Hottiger *et al.*, 1994). Trehalose has also been found to reduce the formation of heat-induced protein aggregates. Labile enzymes dried in the presence of trehalose can be reconstituted after prolonged storage with no loss of activity and will withstand

exposure to extreme heat (Colaco *et al.*, 1992). It is possible that this disaccharide also promotes renaturation of stress-damaged enzymes. Experiments have shown that disruption of *Saccharomyces cerevisiae* genes encoding sub-units of trehalose-6-phosphatase can be used to prevent the organism accumulating trehalose. This mutation leads to a significant reduction in thermotolerance (Devirgilio *et al.*, 1994). All of this evidence points to the importance of trehalose in protection against stress. However, other researchers have produced mutants of *Saccharomyces cerevisiae* which are deficient in a gene which encodes the trehalase enzyme responsible for degrading trehalose. The absence of these enzymes leads to the accumulation of high levels of trehalose, but the mutants show a low thermotolerance. A ubiquitin deficient mutant was also investigated during this study which showed low trehalose concentrations but high thermotolerance during exponential growth. In this case it was proposed that the constitutively produced heat shock proteins were probably responsible for mediating thermotolerance (Nwaka *et al.*, 1994).

In yeast, trehalose accumulation has been shown to be regulated by HSP70. Strains which lack some of *hsp70* genes overproduce trehalose and show slower degradation rates on removal of the heat shock. Smaller effects are exerted by HSP104 and HSP90 (Cheng *et al.*, 1993).

Glycerols and erythritols are also known to play an important part in adapting a number of entomopathogenic fungi, including *M. anisopliae*, to water stress (Hallsworth and Magan, 1995). Glycerol is accumulated intracellularly by the induction of glycerol-3-phosphate dehydrogenase in response to osmotic stress, this leads to an increase in solutes within the cell and eventually a restoration of balance in osmotic pressures intra- and extra-cellularly (Albertyn *et al.*, 1994). It has also been shown that if protein synthesis is inhibited during heat shock in *Saccharomyces cerevisiae*, thus preventing HSP synthesis, there is still an induction of some thermotolerance which correlates with an increase in glycerol production under these conditions. Under these conditions

trehalose accumulation was also depressed indicating that this process also requires protein synthesis (Lewis *et al.*, 1995). Melanin production can be important in protection against UV and temperature extremes (Bell and Wheeler, 1986).

4.1.8 HSPs and infection

A number of studies implicate the role of HSPs in fungal infections of humans. Firstly, heat shock proteins in a number of human fungal pathogens, such as *Candida albicans* and *Histoplasma capsulatum*, are produced very early on in the infection cycle. They are thought to be important in adapting the organism to the stresses of a new environment (Maresca *et al.*, 1994).

Secondly, there is strong evidence to suggest that HSPs play an important role later on in human fungal infections. In *Histoplasma capsulatum* the ability to produce heat shock proteins can even be correlated to virulence. A 45kDa fragment of HSP90 in *Candida albicans* systemic infections is the immuno-dominant antigen and is far more likely to be fatal in those patients unable to produce antibodies against it. It is believed that the pathogens HSPs are not secreted but are released when the cell dies and lyses. The pathogens HSPs are thought to be harmful because they bind to the hosts serum proteins and cause them to malfunction by effecting protein folding, inhibiting interactions or preventing degradation (Matthews and Burnie, 1992). A murine monoclonal antibody raised against an epitope of HSP90 (LKVIRK) and given prophylactically reduced mortality in a mouse model of invasive candidosis (Matthews *et al.*, 1991). Further evidence for the importance of HSPs in infection is the fact that mice can be immunised against tuberculosis by vaccination with transgenic antigen-presenting cells which contain the *Mycobacterium leprae hsp65* gene (Silva and Lowerie, 1994). It is impossible for microbes to evolve immunity to such antibody treatment as the HSPs are essential. Such antibodies should provide not only protection against the original target organism, but also cross-protection against other organisms as they are such highly conserved molecules. It is proposed that these antibodies protect

the host by binding to the active site, preventing the foreign HSP from binding serum proteins. Interestingly, the amounts of heat shock protein present in breast cancer cells have been found to be related to aggression (HSP70) and drug resistance (HSP27) (Mohsenzadeh *et al.*, 1994).

4.1.9 Heat Shock Proteins and *Metarhizium flavoviride.*

Heat shock proteins are of interest in *M. flavoviride* for three reasons. Firstly, HSPs may be important in protecting the fungus from the temperature changes it is likely to face on application in the field. This is particularly important in the case of *M. flavoviride* which is being developed for use against locusts and grasshoppers that can exist in very hot and arid conditions. It is possible that a mild heat shock prior to application may improve both the ability of the fungus to survive at the high temperatures in the field and may also improve its ability to infect. Heat shock of *Bacillus spp.* spores has been shown to improve their ability to germinate at higher temperatures (Sedlak *et al.*, 1993). Heat treatment of another entomopathogenic fungus, *Beauveria bassiana*, has been investigated as a possible mechanism of inducing germination, but was found to have no effect (Smith and Grula, 1981).

HSPs may also be important in adapting the fungi to the stresses it faces on encountering the host such as temporary nutrient limitation. In *M. anisopliae* one stress protein (not an HSP) has already been identified as being produced early on during the formation of infection structures and during nutrient depravation (St. Leger *et al.*, 1992). Regulation of PR1 (thought to be a major pathogenicity determinant) in a number of entomopathogenic fungi including *M. anisopliae* occurs during starvation, one of the stresses known to induce HSP production. Growing *M. anisopliae* on cockroach cuticle media has also been shown to repress and induce a number of different mRNA species which is a typical response during the induction of HSPs (St. Leger *et al.*, 1995).

Finally, HSPs could be virulence factors in entomopathogenic fungi, as they are in human pathogenic fungi; by binding proteins in the host's haemolymph and causing their inactivation or malfunction HSPs could contribute to the death of the insect.

4.1.10 Heat Shock Proteins in *Schistocerca gregaria.*

It is worth considering the possible importance of the locusts HSPs, although they have not been investigated during this study. Heat shock proteins may protect *S. gregaria* from the invading fungi. It has been shown that rats subjected to whole body hypothermia before the induction of lung damage will have significantly reduced mortality and lung damage (Villar *et al.*, 1993). This phenomenon is accompanied by a significant increase in the translation and transcription of *hsp70.* If the prior induction of HSPs in locusts has a similar protective effect against *M. flavoviride* infection it may have implications for timing application of the formulations.

4.1.11 Aims

The aims of this work were firstly to investigate the induction of thermotolerance in *M. flavoviride* spores in terms of their subsequent ability to germinate and form colonies. Secondly, to look at the roles of HSP70 and HSP90 in both *M. flavoviride* isolate IMI 330189 and *M. anisopliae* isolate ME1 using both biochemical and molecular means.

4.2 Materials and Methods

4.2.1 Materials

Media constituents were all from Lab M unless otherwise stated. DNA modifying enzymes and antibiotics were supplied by NBL Gene Science Ltd. Radioactive isotopes were supplied by ICN Ltd. Other chemicals were supplied by Sigma Chemical Company Ltd. unless otherwise stated.

4.2.2 Methods

4.2.2.1 Thermotolerance

Conidial suspensions of *M. flavoviride* isolate IMI 330189 were prepared in 0.04% Tween 80 as described in Chapter 2. Suspensions were diluted to 6.2×10^3 conidia ml^{-1} for colony forming experiments and 3×10^6 conidia ml^{-1} for germination experiments. 0.5ml of these suspensions were dispensed into sterile 0.5ml microcentrifuge tubes, which were then immediately transferred to water baths at the required temperature. After heat shock 3 replicates of 150µl of each sample were plated onto 90mm diameter quarter strength SDA plates for conidial survival experiments or onto 1.5% water agar plates for germination experiments. The plates were then incubated at 27°C in the dark for 24, 48 or 72 hours before the number of colony forming units or percentage of germinating spores was assessed.

4.2.2.2 Protein production

4.2.2.2.1 Liquid culture of *Metarhizium spp.*

For liquid cultures, 100ml of the Czapek Dox complete media (Appendix 1) was autoclaved in 250ml conical flasks. These were inoculated with 3×10^7 conidia and left to grow for 72 hours at 27°C at 120 rpm and in the dark unless otherwise stated.

Heat shock was applied by placing the cultures in a shaking water bath at the required temperature for 60 minutes unless otherwise stated.

4.2.2.2.2 Preparation of locust cuticle

Locust cuticle was a gift from J. Gillespie. It was prepared using the method of Anderson (1980). Approximately 100 locust were killed by freezing at -20°C and after defrosting were homogenized in a Waring blender in 1l of 1% potassium tetraborate. The material was poured through a 1mm pore size sieve and the material remaining was further homogenized and then washed several times in 1% potassium tetraborate. The cuticle was then stirred in 2l of 1% potassium tetraborate overnight, filtered, washed in distilled water and air dried. The cuticle was next milled through a 0.5mm sieve in a Glen Creston-Ball Mill (DEH 48) and washed again in 1% potassium tetraborate and then in distilled water. Finally the cuticle was left in water and impurities removed by decanting off the supernatant once the cuticle had settled to the bottom. 1% locust cuticle in muslin bags (to aid separation of mycelia) was added to basal salts media (Appendix 1) which had already been autoclaved and was then re-autoclaved at 15lb in^2 for 5 minutes to minimize solubilisation of cuticle components.

4.2.2.2.3 Starvation and transfer experiments

For experiments in which it was necessary to transfer mycelium from one flask to another, mycelium was poured through a double layer of sterile muslin filter and washed extensively with sterile distilled water. A sterile spatula was then used to transfer this mycelium to a fresh flask of media.

4.2.2.2.4 Effects of heat shock on mycelium

After heat shock 3 replicate samples of 1ml of culture were removed and aseptically transferred to 100ml of fresh Czapek Dox complete media. Cultures were incubated for a further 48 hours at 27°C and 120rpm in the dark. After this time the cultures were filtered through a Buchner funnel containing pre-weighed Whatman filter paper 1 which had been dried at 60°C until the weight was consistent on two consecutive days. The mycelium was extensively washed with sterile distilled water and then left in a drying oven at 60°C until the weight was consistent on two consecutive days. The initial

weight of the filter paper was subtracted from this weight in order to obtain a dry weight for the fungal biomass.

4.2.2.2.5 Protein extraction from *Metarhizium spp.*

After heat shock mycelial cultures were filtered through sterile Whatman filter paper number 1 using a Buchner funnel and then washed with sterile water (which was at the same temperature as the heat shock being applied). The mycelia was then transferred to pre-chilled mortar and liquid nitrogen poured on top. The mycelia was ground to a fine powder using a pestle. Approximately 500mg of this tissue was put into a sterile 1.5 ml microcentrifuge tube and 1ml of protein extraction buffer (Appendix 7) was added. The mixture was thoroughly vortexed for about 1 minute using a Fisons whirlimixer and then spun in a microcentrifuge at 11600×g in a Microcentuar microcentrifuge for 10 minutes. The supernatant was passed through an Acrodisc PF 0.8/0.2μm Supor.

4.2.2.2.6 Haemolymph extractions

Adult locust were infected and maintained as described in the methods in Chapter 3. Haemolymph was extracted from infected and non-infected insects on each day of infection. A pin was used to puncture the cuticle on the thorax under the hind leg. Haemolymph was extracted from this hole using a 10μl capillary tube. The haemolymph was then placed into a sterile microcentrifuge tube containing a crystal of phenylthiourea and 40μl of anticoagulation buffer (Gillespie, 1995). Samples were immediately stored at -20°C until required.

4.2.2.2.7 SDS-PAGE

SDS-PAGE was performed using the BIO-RAD mini protean II. Plates, spacers and combs were cleaned with detergent, rinsed twice in tap water and once in distilled water and then rinsed in 70% ethanol and allowed to air dry. The gel units were then assembled in the casting stand and 3.5ml of resolving gel (Table 4-2) was poured into each. Water saturated butanol was then used to overlay the gel, the acrylamide was allowed to polymerise for 1 hour. The overlay was then removed and the top of the gel

rinsed in resolving gel buffer stock (Appendix 7). The stacking gel (Table 4-3) was then prepared and poured onto the resolving gel to the top of the lower plate, the comb was inserted and the gel was left to polymerise for 45 minutes. The comb was then removed and the wells rinsed with 1× running buffer (Appendix 7).

The quantity of protein in each sample was measured using the BIO-RAD protein assay kit micro-assay procedure described in the instructions. For each sample about

50µg of protein was mixed with an equal volume of 2× sample loading buffer (Appendix 7). If necessary, dilute samples were lyophilised and then loaded in 15µl of 1× sample loading buffer. The samples were heated to 99°C for 3 minutes in Hybaid thermocycler, placed on ice and then centrifuged at 13,000rpm for 60 seconds before being loaded into the wells.

Table 4-2. Resolving gel recipes

	Final gel concentration				
	5%	**7.5%**	**10%**	**12.5%**	**15%**
Monomer solution*	1670µl	2500µl	3300µl	4200µl	5000µl
8× resolving gel buffer[1]	1250µl	1250µl	1250µl	1250µl	1250µl
10% SDS	100µl	100µl	100µl	100µl	100µl
MilliQ water	6950µl	6150µl	5250µl	4450µl	3650µl
10% Ammonium persulphate	50µl	50µl	50µl	50µl	50µl
TEMED	3.3µl	3.3µl	3.3µl	3.3µl	3.3µl

Table 4-3. Stacking gel recipe

Monomer solution*	880µl
8× stacking gel buffer[1]	1660µl
10% SDS	66µl
MilliQ water	4060µl
10% Ammonium persulphate	33.4µl
TEMED	3.3µl

* Protogel (30% acrylamide, 0.8% bisacrylamide) from National Diagnostics. 1 - refer to Appendix 7 for recipe.

The BIO-RAD mini protean II was then assembled with the gels as described in the manual. The upper and lower chambers were filled with 1× running buffer, bubbles were removed from the base of the gel with a bent glass pipette. Samples were loaded into the wells using a 25µl Hamilton syringe. Any empty lanes were loaded with 1× sample buffer containing no protein. The assembly was then connected to a power supply and run at a constant 150V until the dye front reached the bottom of the gel.

The gel was then removed from the assembly and a small nick made in the bottom left hand corner to allow orientation of the gel. The gel was placed into Coomassie blue staining solution (Appendix 7) overnight. Excess Commassie blue stain was removed from the gels by repeated changes of destain solution (Appendix 7) until the background was clear, the gels were photographed if necessary and then dried under vacuum with a gel dryer.

4.2.2.2.8 Gel drying

Protein and sequencing gels were placed onto Whatman 1 filter paper and covered with Saran wrap. These were placed on the gel dryer and dried at 60°C under vacuum for 2 hours.

4.2.2.2.9 ^{35}S Labeling of *Metarhizium spp.* proteins *in vivo*

10ml of samples of mycelium were removed from three day old cultures and placed into 25ml sterile plastic universals. These were placed in a shaking water bath at the required heat shock temperature for 15 minutes. After this time 5µCi of Trans ^{35}S-label (L-Methionine,^{35}[S], L-cystiene,^{35}[S], Sp. Act. >1000 Ci/mmol, ICN) was added to the cultures and they were incubated at the same temperature in a Innova 4000 incubator shaker at 120rpm for a further 45 minutes. Proteins were extracted as described previously.

4.2.2.2.10 ^{35}S Labeling of proteins from haemolymph

Adult male locusts, more than two weeks after moulting, were infected with 5µl of 3× 10^6 conidia ml^{-1} *M. flavoviride* isolate IMI 330189 in Shell ondina el oil and incubated

as described in Chapter 2. Control insects were inoculated with 5µl of oil alone and treated in the same way. Insects were removed every day over the course of the infection and injected with 5µCi of Trans ^{35}S-methionine label before being returned to the incubator for an hour. The insects were then bled as described previously.

4.2.2.2.11 Autoradiography

Total and TCA precipitable counts were determined for all ^{35} S-methionine experiments. For total counts 5µl of protein sample were added to 10ml of LKB OptiPhase 'safe' scintillation liquid in scintillation vials. For TCA precipitable counts 5µl of sample was mixed with 1ml of ice cold 25% TCA in a sterile 1.5ml microcentrifuge tube and left on ice for 30 minutes. The sample was then placed in a microcentrifuge and spun at 13000 rpm for ten minutes. The supernatant was then removed and 1ml of ice cold 5% TCA was added. The sample was vortexed for 30 seconds and then spun again for 10 minutes at 13000rpm before removing the supernatant. This step was repeated three times. Finally the pellet was air dried before being solubilised in 20µl of water and added to 10ml of scintillant in a scintillation vial. The number of dpm were determined using a LKB Wallac 1217 Rackbeta liquid scintillation counter.

4.2.2.2.12 Western blotting

SDS-PAGE gels were equilibrated in Towbin buffer (Appendix 7) at 4°C for 20 minutes after being run as described previously. These gels were blotted onto nitrocellulose membranes (which had also been equilibrated for an hour at 4°C in Towbin buffer) using the BIO-RAD mini trans-blot. Blotting was carried out overnight at 30V at 4°C as described in the manual.

After transfer, the nitrocellulose membranes were blocked by transferring them to TBS (Appendix 7) with 5% milk powder (Marvel). The filters were then left gently shaking for an hour at room temperature. After giving the membranes two 5 minute washes in TTBS (Appendix 7), the membranes were transferred into a 1:5,000 dilution of primary antibody (monoclonal anti-HSP70 from Affinity Bioreagents Inc.) in TTBS with 3%

milk powder. Although this antibody was not raised against *M. flavoviride*, it was raised against a yeast and company literature showed it to be highly cross-reactive with a range of HSP70s from organisms as diverse as plants and animals. It was therefore considered suitable for these experiments. The membranes were again left gently shaking at room temperature for a further hour. The membrane was given two 5 minute washes in TTBS. The nitrocellulose membrane was then put into a 1:30,000 dilution of secondary antibody (alkaline phophatase conjugated rabbit anti-mouse IgG) in TTBS with 3% milk powder for another hour. After this the membrane was given a final two 5 minute washes in TTBS and then rinsed with a large volume of TBS.

To develop the Western blot, 1ml of 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrezolium liquid substrate was placed on the membrane until bands were observed and was then washed off in three changes of distilled water. Blots were photographed, dried and stored in foil.

4.2.2.3 Molecular cloning

4.2.2.3.1 Fungal DNA isolation

DNA was isolated from *M. anisopliae* isolate ME1 and from *M. flavoviride* isolate IMI 330189 from a method adapted from Raeder and Broda (1985). Mycelia were harvested, frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. This was transferred to a solvent resistant centrifuge tube containing 10ml of fungal extraction buffer (Appendix 3). Protein was removed by adding an equal volume of phenol:chloroform (7:3) equilibrated with fungal extraction buffer and mixing gently for 30 minutes. The tubes were then centrifuged for 1h at 10000×g and 10°C. The aqueous phase was transferred to a clean tube and the RNA removed by digestion with DNase free RNase A (10μg ml^{-1}) for 1h at 37°C. RNase A and proteins were removed by mixing with an equal volume of chloroform:isoamyl alcohol (24:1) for 15 minutes followed by centrifugation at 10000×g for 15 minutes at 10°C . DNA was precipitated from the aqueous phase in a clean centrifuge tube by the addition of 0.6 volumes of

isopropanol and centrifuged at 7000×g for 15 minutes at 20°C . DNA was washed twice with ice cold 70% ethanol to remove traces of salt before drying at room temperature for 30 minutes. The DNA was then re-dissolved in 1 - 5ml of TE (Appendix 3). The DNA concentration and purity were checked with the formulae below:

concentration of DNA stock ($\mu g\ ml^{-1}$) = $50 \times A_{260} \times$ dilution factor

The purity of the DNA was estimated by calculating the A_{260}/A_{280} ratio, pure DNA has a ratio of approximately 1.7 - 2.0.

4.2.2.3.2 Agarose gels

Agarose gels were prepared in 1×TBE (Appendix 3). The percentage of agarose added to the gel was dependent on the fragment sizes being isolated (Table 4-4).

Table 4-4. The percentage of agarose gels necessary to separate DNA fragments of different sizes.

Size of fragment (kb)	**Percentage agarose** (w/vol.)
1.0 - 20	0.6
0.8 - 10	0.7
0.5 - 7	0.9
0.4 - 6	1.2

The required amount of agarose was added to 1×TBE buffer and heated in the microwave until all the agarose had dissolved. The solution was then cooled to about 50°C and 0.5$\mu g\ ml^{-1}$ ethidium bromide was added. The solution was then poured into the gel tray and allow to set for 30 minutes.

Samples were mixed with appropriate volume of 6× loading buffer (Appendix 3) and loaded onto agarose gels. These were electrophoresed at between 1-5V cm^{-1} in a tank of 1× TBE buffer until the bromophenol blue had reached the bottom of the gel. The gels were examined and photographed under UV illumination.

4.2.2.3.3 Restriction digests

Restriction digests were carried out with the DNA sample diluted to 17µl with sterile distilled water. 2µl of the appropriate 10× restriction buffer were added along with 1unit of each restriction enzyme. The reactions were incubated at 37°C for between 4-18 hours depending on the final use of the sample.

4.2.2.3.4 Polymerase Chain Reaction

4.2.2.3.4.1 Primer design

Primers were designed by aligning several known fungal and yeast *hsp70* and *hsp90* deduced amino acid sequences from the gcg database and identifying regions which showed a high level of homology (in most cases 100%). Primers were also designed to have cloning sites at the 5' end.

4.2.2.3.4.2 PCR conditions

All polymerase chain reactions contained the same concentrations of reagents, primers and DNA, only the temperature was altered to achieve single products in reactions. All plasticware used for PCR was handled only with gloved hands in a laminar flow hood.

The reactions were prepared in 50µl aliquots in 0.5ml microcentrifuge tubes according to the recipe given in Appendix 4. 50µl of sterile mineral oil was overlaid on the top to prevent evaporation. The reactions were carried out in Hybaid OmniGene PCR machines with the conditions listed in Table 4-5.

Table 4-5. PCR conditions used in the amplification of *hsp70* and *hsp90* fragments from *Metarhizium anisopliae* ME1 and *Metarhizium flavoviride* IMI 330189 genomic DNA.

Purpose of Step	Time	Temperature
Denaturing	1 minute	94 °C
Annealing	1 minute	60 °C (*hsp70*)
		55 °C (*hsp90*)
Extending	1 minute	72 °C
	This cycle was repeated 35 times.	
Extending	5 minutes	72 °C

4.2.2.3.5 Cleaning and purification of DNA

Specific gel bands were purified using the QIAGEN QIAquick gel extraction kit™. This DNA was then cleaned using the USB MicroSpin™ column.

4.2.2.3.6 Preparing recombinant plasmid Ptz19r

Restriction reactions were set up containing single clean PCR products and the restriction enzymes EcoRI and BamHI. 0.1μg of plasmid DNA was mixed with 0.1μg of insert DNA and sterile distilled water was used to make the volume up to 7.5μl in total. The DNA was warmed to 45°C for 5 minutes and then put on ice. 1μl of 10× ligase buffer, 0.1U of T4 DNA ligase and 1μl of 5mM ATP were added to the DNA, control reactions contained either the plasmid or foreign DNA alone. The mixture was incubated overnight at 14°C.

4.2.2.3.7 Transforming *Escherichia coli* with plasmid DNA

E. coli strain JM109 from a single colony were added to 5ml of sterile LB (Appendix 1) in a universal and incubated at 37°C and 200rpm overnight. 0.5ml of this culture was removed and used to inoculate a 100ml culture of YT (Appendix 1). This culture was

then returned to the 37°C incubator for another two hours. The cells were then removed and centrifuged at 10000×g for 2 minutes at 4°C. The cells were kept on ice from this stage onwards. The supernatant was discarded and 10ml of ice cold 50mM $CaCl_2$ were added and the cells re-suspended. After 20 minutes on ice the cells were centrifuged again at 10000×g for 2 minutes at 4°C. The supernatant was discarded and 0.5ml of ice cold 50mM $CaCl_2$ added and the cells re-suspended. 100µl of cell suspension were added to each of the recombinant plasmid ligations. The cells were left on ice for 90 minutes and then heat shocked at 42°C for 2 minutes. The cells were transferred to 5ml of sterile YT media in a universal and incubated at 37°C for 2 hours. These were then plated onto transformation plates (Appendix 1), inverted and incubated at 37°C overnight. Transformed recombinant plasmids were identified by their white colouring.

4.2.2.3.8 Plasmid isolation

White transformed colonies which contained recombinant plasmids were streaked onto fresh transformation plates and allowed to grow up overnight at 37°C. These bacteria were used to inoculate 5ml of sterile LB media which had been supplemented with 10µg ml^{-1} ampicillin. These cultures were grown up overnight at 37°C and 200rpm. 1ml of these cultures was placed in a 1.5ml microcentrifuge tube and spun at 10,000×g for 3 minutes in a microcentrifuge, the supernatant was then discarded and the pellet re-suspended in 100µl of GTE (Appendix 3). 200µl of NaOH/SDS were added and the tubes left for 5 minutes at room temperature. 150µl of KAcF were then added and gently mixed by inversion, this solution was left on ice for a further 5 minutes. 200µl of phenol were then added followed by 200µl of 24:1 chloroform:isoamyl alcohol. The contents of the microcentrifuge tubes were mixed thoroughly using a whirlimixer and then centrifuged at 10,000×g for 5 minutes. The top aqueous layer was transferred to a fresh 1.5ml microcentrifuge tube and a further 200µl of 24:1 chloroform:isoamyl alcohol were added, the contents of the tube were again thoroughly mixed and centrifuged again at 10,000×g for 3 minutes. The top aqueous layer was then transferred to another sterile microcentrifuge tube and 100µl of 10M ammonium acetate

were added. The rest of the microcentrifuge tube was filled with ice cold 95% ethanol, mixed gently by inversion and centrifuged at 4°C for 15 minutes at 10,000×g. The supernatant was discarded and the pellet washed with 1ml of ice cold 70% ethanol. The solution was centrifuged again for 7.5 minutes at 10,000×g. The supernatant was then removed and the DNA pellet lyophilised for 5-10 minutes. The pellet was then re-suspended in 25µl of TE buffer.

4.2.2.3.9 Analyses of recombinant plasmid

The plasmid was restricted with the restriction enzyme(s) originally used to cut both the plasmid and the fragment. The samples were then loaded onto agarose gels and electrophoresed (including cut and uncut plasmid references). The resulting banding pattern gave two bands, one of 2.8 kb (the plasmid) and one for the inserted fragment.

4.2.2.3.10 Southern blots

Gels for blotting were trimmed to remove wells and any empty lanes. The gels were soaked 0.25M HCl for 20 minutes, then in denaturing solution (Appendix 3) for 30 minutes and finally in neutralizing solution (Appendix 3) for 30 minutes. A sponge wick was placed in the bottom of a glass tray and soaked in 20× SSC (Appendix 3) on top of this were placed 6 layers of 3mm Whatman chromatography paper which were also soaked in 20× SSC. The gel was placed on top of this and surrounded with saran wrap to ensure transfer occurred only through the gel. A marked piece of Hybond N membrane cut to exactly the same size as the gel was placed on top of this followed by a piece of 3mm chromatography paper which had been soaked in 20× SSC and then a piece of dry 3mm chromatography paper. A two inch layer of green paper towels was put on top of this and finally a glass plate with a 500g weight.

4.2.2.3.11 UV cross-linking

DNA and RNA were fixed to nylon membranes which had been used in Southern and Northern blotting by drying the membrane thoroughly and then wrapping it in Saran

wrap. The membrane was then exposed to UV for 5 minutes. Membranes were stored at -20°C until required.

4.2.2.3.12 Labeling reactions

Synthetic oligonucleotides were labeled with [γ-^{32}P] dCTP. 1µl of the oligonucleotide (10pmol) was mixed with 2µl of 10× T4 polynucleotide kinase buffer, 5µl of [γ-^{32}P] dCTP (~ 5000 Ci/mM) and 11.4µl of water. 1µl of T4 polynucleotide kinase was added before incubation at 37°C for 45 minutes.

Genomic DNA and PCR products were labeled with [α-^{32}P] dCTP using the Pharmacia Biotech oligonucleotide labeling kit. 25 - 50ng of denatured oligonucleotide dissolved in a total volume of 34µl of TE buffer (pH 8.0) were heated to 95°C for 2 minutes to denature and then put on ice for 2 minutes. 2µl of T4 polynucleotide kinase buffer was then added followed by 5µl of [α-^{32}P] dCTP (~ 3000Ci/mM). 1µl of Klenow fragment was added to the reaction and the mixture was briefly centrifuged. This was then incubated at 37°C for 60 minutes before being used.

Incorporation of the probe was checked by spotting a small amount onto a cellulose strip and placing it into a beaker of distilled water. This was left until the solvent front had reached within 1cm of the top of the strip before being dried and placed on film for up to 1 hour before being developed.

4.2.2.3.13 Probing Southern and Northern blots

Nitrocellulose filters from Southern and Northern blots were incubated at the required temperature (dependent on the specificity of the probe) in pre-hybridisation solution (Appendix 3) for 4 hours with gentle agitation to prevent non-specific binding. All but 1µl of the radioactively labeled probe was added to the solution and it was left in the incubator for 18 hours. The solution was then poured off the filters and they were washed with an appropriate dilution of 20× SSC (dependent on the stringency required which was estimated according to the specificity of the probe and the dpi of the filter).

4.2.2.3.14 DNA sequencing

All reactions for manual sequencing were performed with the USB Sequenase® version 2.0 DNA sequencing kit. Automated sequencing was performed on an ABI automated sequencer.

Sequencing plates were cleaned with detergent and thoroughly rinsed and dried. These plates were then washed with distilled water, then 100% ethanol and finally acetone. The plate with the lugs was covered with silane and then allowed to dry for 5 minutes. Excess silane was washed off with sterile distilled water. The bottom plate was leveled horizontally with a spirit level and spacers were placed on either side of the plate. 50ml of acrylamide gel was prepared with NBL sequencing gel acrylamide and this was poured onto the centre of the bottom plate. The plate with lugs on was then gradually lowered into place (silane side down) and after aligning was clamped into position. A comb was pushed into the top and the gel was allowed to set for 30 minutes. After this time the gel was cleaned up and placed into the tank with 1× TBE in the upper and lower buffer chambers. The gel was pre-electrophoresed for 60 minutes at 45W. Samples were loaded and the run time varied depending on which sequence was required.

4.2.2.3.15 Sequence analysis

Sequences were analysed using the gcg database on gnome.

4.2.2.3.16 λ Library construction

The *M. anisopliae* ME1 genomic library was prepared by I.C. Paterson (Paterson, 1992). DNA was partially digested with Mbo1, size fractionated on a sucrose gradient and fragments of 15 - 20kb were cloned into BamHI digested λ EMBL3 arms and packaged.

4.2.2.3.17 Preparation of plating cells.

E. coli cells (strain P2392) were removed from storage at -20°C and a loopful was streaked over an LB agar plate. These cells were allowed to grow at 37°C in the dark

overnight. A single colony of the cells was picked and used to inoculate 100ml of LB liquid media supplemented with 0.2% maltose. The culture was incubated at 37°C and 200rpm overnight. Cells were then spun down at 4000×g for 10 minutes and re-suspended in sterile 0.01M $MgSO_4$ to an OD_{600} of 2.0. These cells were stored at 4°C and used for up to three weeks after preparation.

4.2.2.3.18 Titre of λ library

A series of 10× dilutions were made in SM buffer (Appendix 5) from the *M. anisopliae* ME1 λ library. 0.1ml of these dilutions were mixed with 0.1ml of plating cells and incubated at 37°C for 20 minutes. After this time the cells were added to 5ml of molten top agarose (LB with 0.8% agarose) which was at 47°C and quickly poured over LB agar plates which had been incubated at 37°C for 1 hour. The agarose layer was allowed to set before the plates were inverted and incubated at 37°C overnight. The number of pfus were recorded for each plate and an estimate of the titre was made.

4.2.2.3.19 Preparation of λ plaques

LB agar plates were prepared in $22cm^2$ square Petri dishes and allowed to dry thoroughly. Two sets of dilutions of the λ library were prepared which contained approximately 5000 and 2500 pfu respectively. 1.5ml of each λ dilution was prepared in SM buffer and added to 1.5ml of plating cells and incubated at 37°C for 20 minutes as described previously. These cells were then added to 75ml of top agarose which had been incubated at 47°C and mixed rapidly before being poured over the top of the LB agar. The plates were left to set for 10 minutes before being inverted and incubated at 37°C overnight. The number of pfus were recorded.

4.2.2.3.20 Transfer of λ DNA to a nylon membrane

Two nylon membranes were cut and marked for each of the four plates containing λ plaques. In each case the first nylon membrane was placed carefully on the top of the agarose layer and its orientation marked with pin holes and ink, it was left in place for one minute. The second nylon filter was left in place for 5 minutes. After this the

membranes were transferred DNA side up onto filter papers soaked in denaturing solution and left for 5 minutes. They were then transferred to filter papers soaked in neutralizing solution for a further 5 minutes and finally transferred to filter papers soaked in 2× SSC and left again for 5 minutes. The membranes were left until thoroughly dry and then bound to the membrane by UV cross-linking (as described earlier).

4.2.2.3.21 Treatment of glassware, plastics and solutions for RNA work

All glassware to be used with RNA was cleaned thoroughly with detergent and rinsed with de-ionised water, it was then oven baked at 240°C overnight.

Disposable plastic-ware was reserved solely for use with RNA and was handled only with gloves. Tips and microcentrifuge tubes were transferred to pre-treated glass beakers (as described above) before being autoclaved at 120°C, 15 lb in^2 for 30 minutes.

Solutions for use with RNA were treated with 0.1% DEPC (Appendix 6).

4.2.2.3.22 Extraction of RNA

Mycelia were separated from liquid cultures by filtering through a sterile Buchner funnel and washed extensively with sterile distilled water. The mycelia was then transferred to pre-cooled mortar and pestle and ground thoroughly under liquid nitrogen. Approximately 100mg of this was used in the QIAGEN RNeasy™ RNA extraction kit. The RLC lysis buffer (which contained guanidine hydrochloride) was chosen to avoid solidification.

Quantification and purity of the sample were estimated using a spectrophotometer, the amount of RNA is calculated by the formula shown below:

$$\text{concentration of RNA stock } (\mu g\ ml^{-1}) = 40 \times A_{260} \times \text{dilution factor}$$

The purity of the RNA was estimated by calculating the A_{260}/A_{280} ratio, pure RNA has a ratio of between 1.7 - 2.0.

Once extracted the RNA was re-suspended in DEPC treated water and stored at -20°C.

4.2.2.3.23 RNA agarose gels

Formaldehyde gels were prepared by dissolving the appropriate amount of agarose in 3.5 parts water and then cooling to 60°C. One part 12.3M formaldehyde and 1.1 parts 5 × formaldehyde gel running buffer (Appendix 6) were then added and the gel poured immediately.

4.2.2.3.24 Preparation of RNA for running on formaldehyde gel

Before electrophoresis gel tanks were cleaned thoroughly with detergent, rinsed in distilled water and dried with ethanol. The tank was then filled with a 3% solution of H_2O_2 and left for 10 minutes. The tank was then rinsed again with distilled water.

10μg of RNA in a volume of 4.5μl were added to 2μl of 5× formaldehyde gel running buffer, 3.5μl of formaldehyde and 10μl of formamide. Samples were then incubated at 65°C for 15 minutes before adding 2μl of gel loading buffer and loading the sample onto the gel. Gels were run in 1× formaldehyde gel running buffer at 3 - 4V cm^{-1}. Every two hours the buffer was removed, mixed and returned to the gel tank. Lanes to be used in Northern blots were removed and the rest were stained with ethidium bromide.

4.2.2.3.25 rtPCR

RT-PCR was carried out using the Promega Access RT-PCR System.

4.2.2.3.26 Northern blotting

Gels to be used in Northern blotting were not stained with ethidium bromide as this can effect transfer of the RNA. The gel was trimmed and soaked for 20 minutes in 0.05N sodium hydroxide to partially hydrolyse the RNA and improve transfer. The gel was then rinsed in RNAase free water and soaked in 20× SSC for 45 minutes before being blotted as described below.

A solid support was placed in an enamel tray and a piece of 3 MM Whatman paper placed over this as a wick. The dish was filled with 20× SSC almost to the top of the support. The prepared RNA gel was put on top of this and completely surrounded with Saran wrap to ensure all transfer was via the gel. A piece of nylon membrane cut to the same size as the gel was then placed on top of this followed by two pieces of 3 MM filter paper soaked in 2× SSC. Finally a 5cm high stack of paper towels was placed on top of this and the whole apparatus was left over night. Treatment of the membrane after this was as described for Southern blot membranes.

4.3 Results

4.3.1 Thermotolerance

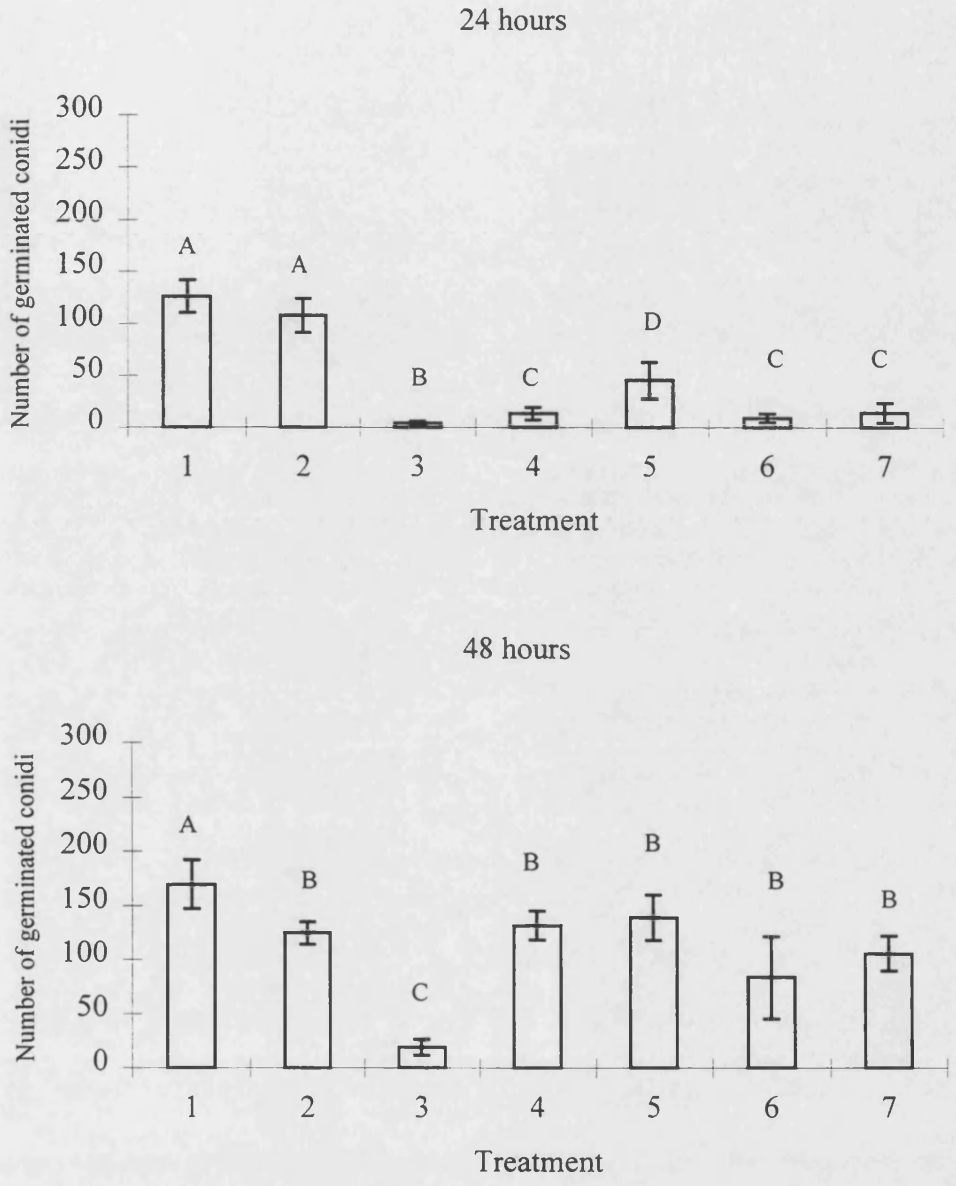
4.3.1.1 The effects of heat shock on the ability of *Metarhizium flavoviride* IMI 330189 conidia to germinate.

Initial experiments were carried out on conidia in an attempt to quantify thermotolerance. *M. flavoviride* isolate IMI 330189 conidia which had been suspended in a solution of 0.04% Tween 80 were heat shocked under different temperature regimes and their ability to germinate was assessed. The results from this experiment are shown in Figure 4-1.

After 24 hours there was no significant difference in the ability of conidia to germinate if they had been exposed to 27°C or 45°C, but if they had been exposed to 50°C for an hour there was significantly less germination. A pre-treatment at 45°C induced significant thermotolerance to a subsequent exposure of 50°C up to 3 hours later, although germination was still significantly less than in those conidia which had been exposed to only 27°C or 45°C. One group showed significantly higher thermotolerance at this stage, those which had been treated at 45°C left for only one hour at 27°C to recover and then heat shocked at 50°C.

The germination of conidia in this experiment appeared to be quite slow, possibly because this investigation used water agar rather than gelatin (as used in Chapter 2). After 48 hours there was significantly more germination in conidia treated at 27°C than those treated at 45°C. Also the germination in samples pretreated at 45°C and then exposed to 50°C showed no significant differences in germination from the conidia which had only been treated at 45°C. The protective effect of pre-exposure to 45°C was maintained during an intervening period of up to 3 hours at 27°C. The conidia which had been immediately exposed to 50°C still showed significantly reduced germination compared to any of the other treatments.

Figure 4-1. The effect of exposing aqueous suspensions of *Metarhizium flavoviride* IMI 330189 conidia to different heat shock regimes on their ability to germinate.



This bar chart illustrates the mean number of germinating conidia (from a total of 300) on a water agar plate after allowing them to germinate for 24 and 48 hours at 27°C in the dark. Each treatment was replicated 5 times with 3 counts made per replicate. The treatments were: **1**, 1 hour at 27°C; **2**, 1 hour at 45°C; **3**, 1 hour at 50°C; **4**, 1 hour at 45°C followed by 1 hour at 50°C; **5**, 1 hour at 45°C then 1 hour at 27°C and 1 hour at 50°C; **6**, 1 hour at 45°C then 2 hours at 27°C and 1 hour at 50°C and **7**, 1 hour at 45°C then 3 hours at 27°C and 1 hour at 50°C. ANOVA and student t-tests were performed between treatments and the letters above the bars indicate groups which are significantly different to each other at α=0.05 (germination at 24 and 48 hours were analysed separately).

This experiment also showed that although pre-exposure to 45°C increased the percentage of germination in conidia subsequently exposed to 50°C, the germination of conidia which had undergone this regime was much slower than for those which had only been exposed to 27 or 45°C.

4.3.1.2 The effect of heat shock on the ability of *Metarhizium flavoviride* IMI 330189 conidia to produce colonies.

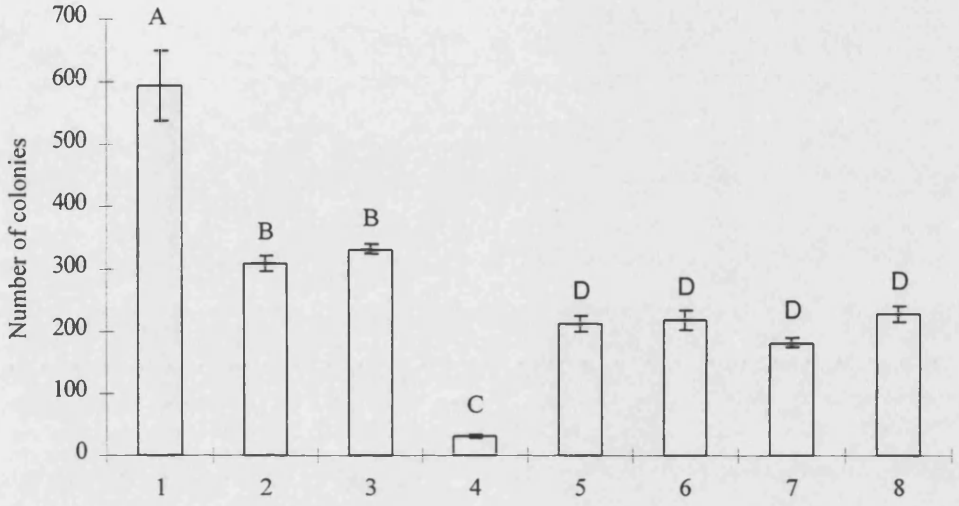
In addition to looking at the effects of heat shock on the ability of *M. flavoviride* conidia to germinate, the effects on their ability to produce colonies was also investigated. Figure 4-2 shows the results of one of these experiments (two replicate trials were carried out with similar results each time).

The results show that exposure to 45°C for an hour lead to a 50% decrease in the number of colony forming units (cfus) when compared to the control conidia exposed to 27°C for one hour. Conidia exposed to 50°C for 1 hour showed a 92% decrease in ability to form colonies. This was not the case if the conidia were first given a pre-treatment at the intermediate temperature of 45°C for an hour before being exposed to the higher temperature of 50°C. This pre-treatment lead to a 4 fold increase in the number of colonies and this protective effect could still be seen in conidia which were exposed to 50°C up to 3 hours after the initial exposure to the intermediate temperature.

Colonies from conidia which had been heat shocked were smaller than those which were not, with colonies treated at 50°C being smaller than those treated at 45°C and these in turn being smaller than those treated at 27°C.

When the experiments were carried out with conidia from plates which had been stored at 4°C for three weeks, it was found that after heat shock treatments at either 45°C or 50 °C for an hour hardly any conidia survived to form colonies (results not shown). Pre-exposure to cold severely reduced thermotolerance in *M. flavoviride*.

Figure 4-2. The effect of exposing aqueous suspensions of *Metarhizium flavoviride* isolate IMI 330189 conidia to different heat shock regimes on their ability to produce colonies.



This bar chart shows the mean number of colonies counted on a quarter strength SDA plate after spreading an estimated 930 treated conidia on to each plate and allowing them to grow for 72 hours at 27 °C in the dark. Each treatment was replicated 6 times. The treatments were: **1**, 1 hour at 27 °C; **2**, 1 hour at 45 °C; **3**, 2 hours at 45 °C, **4**, 1 hour at 50 °C; **5**, 1 hour at 45 °C followed by 1 hour at 50 °C; **6**, 1 hour at 45 °C then 1 hour at 27 °C and 1 hour at 50 °C; **7**, 1 hour at 45 °C then 2 hours at 27 °C and 1 hour at 50 °C and **8**, 1 hour at 45 °C then 3 hours at 27 °C and 1 hour at 50 °C. ANOVA and student t-tests were performed between treatments and the letters above the bars indicate groups which are significantly different from each other.

The effects of heat shock on colony formation (Figure 4-2) differed in certain key respects from the effects of temperature on germination (Figure 4-1). One difference was that the pre-treatment at 45°C was less effective at protecting against a 50°C heat shock when measured in terms of colony formation than germination. This indicates that although conidia may germinate after heat shock a significant proportion of them are unable to form colonies presumably due to defects which are only seen in the later stages of growth.

The smaller colony sizes originating from heat shocked conidia mentioned earlier were probably due to a variety of factors. At 45°C there was no significant delay in germination compared to non-heat-shocked conidia and therefore the smaller colony size observed in these treatments are presumably due to a slower growth rate (Figure 4-1). At 50°C there is a significant delay in germination and the colonies produced after 72 hours are even smaller than those produced by conidia heat shocked at 45°C. It would appear that at 50°C the small colony sizes are due to a combination of delayed germination and a slower growth rate once germinated.

4.3.1.3 Protein production and thermotolerance

In order to investigate further the mechanism of this acquired thermotolerance the experiments were repeated with the addition of 50μg ml^{-1} of the protein synthesis inhibitor, cycloheximide added to the aqueous suspensions. The results are shown in Table 4-6. Cycloheximide had no significant effect on the ability of conidia to form colonies when the solutions were exposed to 27 or 50°C. However, conidia had a significantly reduced ability to withstand 45°C when treated with cycloheximide. Similarly the 1 hour pretreatment at 45°C afforded no protection against subsequent exposure to 50°C for one hour in the presence of cycloheximide. This is illustrated by the fact that there is no significant difference between the number of colonies formed by conidia heat shocked at 50°C in the absence of cycloheximide compared to those given

a pretreatment at 45°C then exposed to 50°C in the presence of cycloheximide (refer to A in Table 4-6).

There was however a small induced thermotolerance in conidia heat shocked in the presence of cycloheximide, that is, there was a significant increase in the number of colony forming units produced at 50°C if the conidia are first exposed to 45°C (reference B in Table 4-6). Possible reasons for this protection are discussed later.

Table 4-6. The effects of the protein synthesis inhibitor cycloheximide on the ability of *Metarhizium flavoviride* isolate IMI 330189 to acquire thermotolerance.

Treatment	Number of colonies (s.e.)		Significance
	- cycloheximide	+ cycloheximide	
27°C 1 hour	880.7 (76.2)	823.3 (22.9)	-
45°C 1 hour	220.7 (10.2)	136.3 (10.9)	+
50°C 1 hour	55.0 (16.0)[A]	18.0 (5.8)[B]	-
45°C 1 hour, 50°C 1 hour	222.3 (34.4)	44.7 (14.9)[A,B]	+

This table illustrates the mean number of colony forming units counted on a quarter strength SDA plate after plating an estimated 930 treated conidia to each plate and allowing them to grow for 72 hours at 27 °C in the dark. Data represents 3 replicates, numbers in brackets show the standard error. The experiment was repeated with essentially similar results each time. Student t-tests were performed between control and cycloheximide treatments and significant differences at the $\alpha = 0.05$ level are shown in the last column. Letters refer to notes made within the text.

4.3.2 Protein production

Further experiments to determine the pattern of protein synthesis under heat shock were all carried out in mycelial cultures, rather than conidia, as these allowed comparisons to be made with published data and provided larger quantities of extractable protein.

4.3.2.1 The effects of heat shock on protein production in *Metarhizium flavoviride* isolate IMI 330189

Initial experiments were carried out to determine temperatures at which mycelial cultures of *M. flavoviride* could survive. Cultures placed in the water bath reached the heat shock temperature within 2 minutes. The effects of heat shock were investigated

by looking at the amount of dry weight 1ml of heat shocked mycelial culture could produce 48 hours after subculture. Analysis of variance revealed no significant differences between the ability of the heat shocked and non-heat shocked fungus to produce biomass (Table 4-7).

Microscopical examinations of the cultures were also carried out and there were no observable differences between cultures heat shocked at 45°C for an hour or those exposed to the control temperature of 27°C.

Table 4-7. Dry weight of mycelia produced after subculturing heat shocked *Metarhizium flavoviride* isolate IMI 330189 cultures.

Temperature of heat shock	Average dry weight of mycelia in grams (±s.e.) after 48 hours subculture
27°C	1.26 (±0.011)
32°C	0.89 (±0.040)
36°C	1.16 (±0.010)
40°C	1.14 (±0.020)
45°C	1.25 (±0.000)

Mycelia from three day old cultures were heat shocked for one hour before 1ml of culture was transferred to 100ml of fresh Czapek Dox complete media and the cultures allowed to grow at 27°C and 120rpm for a further 48 hours. After this time the dry weight of mycelia produced was measured, each dry weight represents the average of three replicates. Student t-tests performed on the data showed no significant differences between any of the treatments.

Having established that the mycelium could survive heat shock of up to 45°C, the production of proteins under heat shock was then investigated. Cultures of *M. flavoviride* isolate IMI 330189 were exposed to heat shock at range of different temperatures for one hour before extracting the intracellular proteins. The quantity of protein in the extract was then calculated. It was noticed that at 45°C there was consistently less protein (≥50%) than in the other heat shocked samples, which may indicate that some autolysis was occurring during the heat shock leading to a lower protein retrieval per unit weight of mycelia. This is surprising when other methods had

revealed no differences in mycelium from heat shocked and non-heat shocked cultures. Extractions at temperatures higher than this had extremely low protein levels.

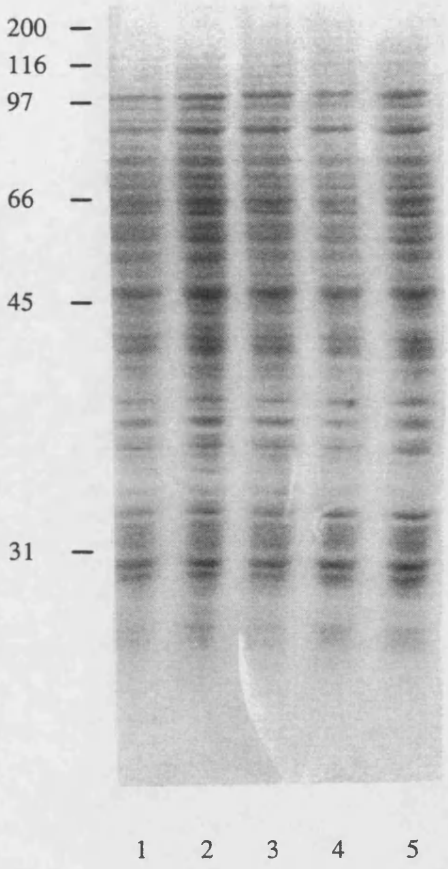
SDS-PAGE gels of these samples were run (with 50μg of protein loaded per lane) and the gels were stained with Coomassie blue to reveal the total proteins present. The results of one of these experiments is shown in Figure 4-3. Analysis of the gels with a densitometer revealed no detectable differences in banding patterns between the treatments.

The effects of duration of heat shock on changes in protein production in *M. flavoviride* were also examined. Mycelial cultures were heat shocked for between 0 and 60 minutes at 45 °C. The intracellular protein extracts were run on a 10% SDS-PAGE gel which was stained with Coomassie blue. No differences were observed in the banding pattern of *M. flavoviride* heat shocked for different durations of time.

4.3.2.2 The effects of heat shock on production of HSP70-like proteins in *Metarhizium flavoviride* isolate IMI 330189

Because of difficulties observing differences in protein production using Coomassie blue stain other methods were adopted. The first of these involved using a commercially available antibody (monoclonal anti-HSP70 from Affinity Bioreagents Inc.) to probe nylon blots of SDS-PAGE protein gels as described in the methods. HSP70 was chosen because of its importance in thermotolerance and role in the infection processes in human fungal pathogens. Company literature showed this antibody to be highly cross-reactive to a range of HSP70s, including those from organisms as diverse as plants and mammals.

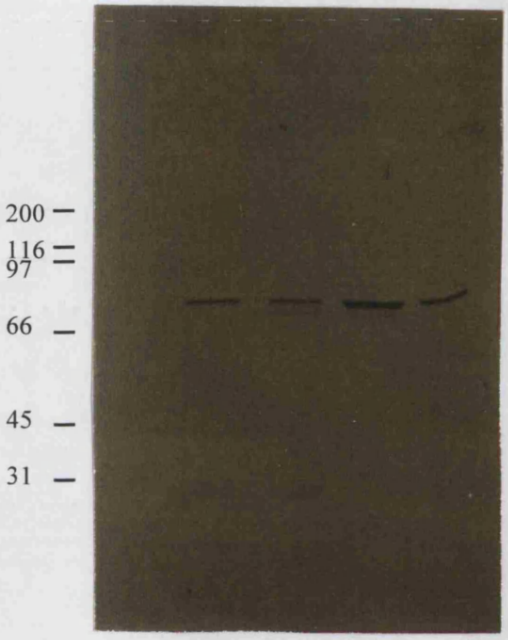
Figure 4-3. A 10% SDS-PAGE gel of intracellular proteins extracted from *Metarhizium flavoviride* isolate IMI 330189 mycelia after one hour heat shock at 27, 32, 36, 40 or 45°C.



The position of the broad range molecular weight markers with the sizes of each protein in kDa are indicated on the left. Lanes 1-5 contain 50 µg of intracellular protein extracted from mycelia that had been heat shocked for one hour at: lane **1**, 27 °C; lane **2**, 32 °C; lane **3**, 36 °C; lane **4**, 40 °C and lane **5**, 45 °C.

Western blots of gels similar to those shown in Figure 4-3 were probed with this antibody and stained. This revealed the presence of three different bands antigenically related to the HSP70 in *M. flavoviride*. There was an intensely staining band of 75kDa, which showed the highest level of heat shock inducibility, and two much fainter bands of 72 and 71kDa. The 72kDa band also appeared to be heat shock inducible at 32 and 36°C. Maximum production of proteins antigenically related HSP70 levels occurred at 32°C. At temperatures higher than this there was a decrease in the amount of 75kDa polypeptide. At 45°C HSP70-like proteins were virtually undetectable. At heat shock temperatures of 32, 36 and 40°C a much lower weight diffuse band appeared at about 40kDa, possibly a break down product of one of the other proteins. This experiment was repeated twice with similar results each time.

Figure 4-4. Western blot of intracellular protein extracts from *Metarhizium flavoviride* IMI 330189 cultures extracted after 1 hours heat shock at 27, 32, 36, 40 or 45°C.



Each of the above lanes was the result of blotting 50 µg of intracellular proteins from *M. flavoviride* IMI 330189 which had been extracted after 1 hours heat shock at; lane **1**, 27°C; lane **2**, 32°C; lane **3**, 36°C; lane **4**, 40°C; lane **5**, 45°C. The size (in kDa) and position of the molecular weight markers are indicated on the left.

4.3.2.3 Proteins produced during heat shock in isolates of *Metarhizium spp.* with varying degrees of virulence against *Schistocerca gregaria.*

A second series of experiments were performed to compare the heat shock responses of different isolates of *Metarhizium spp.* with varying degrees of virulence towards *S. gregaria*, in order to ascertain whether the heat shock response might determine virulence as is suspected in some organisms.

Intracellular protein extracts were made from three different isolates of *Metarhizium* at three different temperatures. Isolates used were the highly virulent *M. anisopliae* isolate ME1 and *M. flavoviride* isolate IMI 330189 and the weakly virulent/avirulent *M. anisopliae* isolate I91 633. Comparisons of the virulence of these three isolates are shown in Table 4-8.

Table 4-8. Comparison of the virulence of three isolates of *Metarhizium spp.* against *Schistocerca gregaria.*

Species	Isolate	Country of origin	Isolated from	Median lethal time for *S. gregaria*
M. anisopliae	ME1	U.S.A.	Coleoptera: Scarabaeoidae	4.82
M. flavoviride	IMI 330189	Niger	Orthoptera: Acrididae	4.41
M. anisopliae	I91 633	Oman	Orthoptera: Gryllidae	*

Data from International Institute of Biological Control. Median lethal times (M.L.T.) were assessed using adult *S. gregaria* 9 - 12 days after ecdysis. Insects were inoculated under the pronotum with 2 µl of suspension containing 3.75×10^7 conidia ml^{-1} in cotton seed oil, mortality was assessed over 12 days and used to calculate the MLT. * indicates that no M.L.T. could be determined for this isolate because there was less than 50% mortality at the end of the experiment.

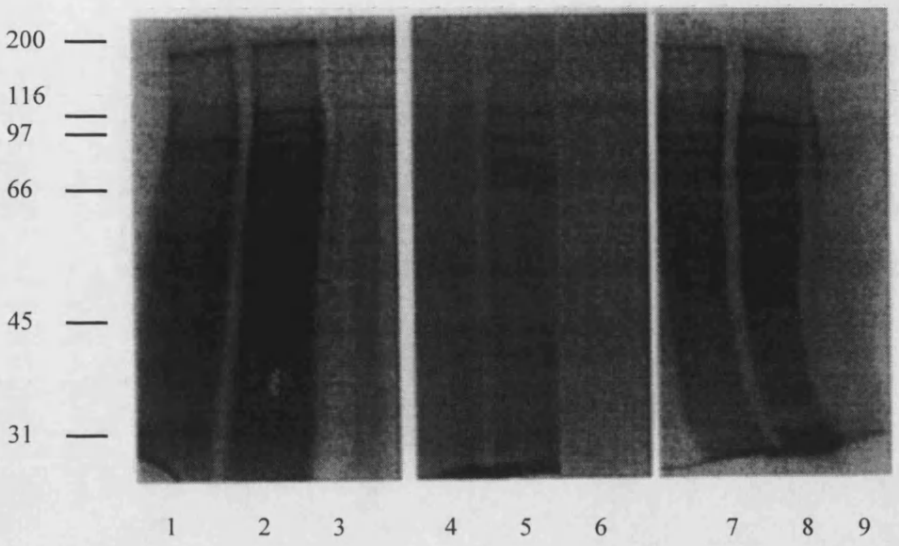
The intracellular proteins from these three isolates were extracted after an hour of heat shock at 27°C, 32°C or 45°C and run on a 10% SDS-PAGE gel. Some differences were observed between the banding patterns produced by the *M. anisopliae* isolates and the *M. flavoviride* isolate. There were very few differences observed between the banding patterns produced by *M. anisopliae* isolate ME1 and I91 633. All three isolates showed

no observable differences in the proteins present in control and heat shocked mycelia. As differences in protein production were difficult to observe with Coomassie blue stain it was decided to try some methods which would enable the observation of only those proteins produced during heat shock and then to look specifically at the quantities of HSP70 homologues present using the commercial antibody raised against HSP70 from *Candida albicans*.

To observe which proteins were produced specifically during heat shock it was necessary to carry out pulse-labeling experiments with radioactive ^{35}S methionine (Figure 4-5). The radioactive methionine was added 15 minutes after the heat shock had begun and intracellular proteins were extracted after an hour as described previously. Equal TCA precipitable dpm of these proteins were run on an SDS-PAGE gel and auto-radiographed.

At 27°C all three isolates of *Metarhzium spp.* produced a wide range of proteins. At 32°C there was a prominent increase in the labeling of a number of different polypeptides in all three isolates. All three isolates showed a markedly increased production of 4 proteins, the most prominent being 106 kDa, with others of 84, 75 and 67kDa. All three isolates also continued to produce most of the normal cellular proteins. At 45°C neither of the two *M. anisopliae* isolates synthesized a detectable amount of TCA radiolabeled protein. *M. flavoviride* however showed an extremely pronounced response, with the arrest of almost all protein synthesis except for the prominent production of one protein at 106kDa. In all three *Metarhizium spp.* isolates it appears that the dominant protein produced in response to heat shock is one of 106kDa.

Figure 4-5. ^{35}S-methionine labeled intracellular proteins extracted from *Metarhzium anisopliae* isolates ME1 and I91 633 and *Metarhizium flavoviride* isolate IMI 330189 mycelia after heat shock for one hour at 27°C, 32°C or 45°C.



The position and size (in kDa)of the molecular weight markers are indicated on the left. Lanes 1,2,4,5 and 7-9 contain 2000 dpm TCA precipitable counts, lanes 3 and 6 contain 100 µl of sample as there was no detectable TCA precipitable count in these samples. Intracellular protein was extracted from *M. flavoviride* IMI 331089 (lanes **4-6**), *M. anisopliae* ME1 (lanes **1-3**) and *M. anisopliae* I91 633 (lanes **7-9**). Heat shock conditions were an hour at 27 °C (lanes **1, 4** and **7**); an hour at 32 °C (lanes **2, 5** and **8**) and an hour at 45 °C (lanes **3, 6** and **9**).

Western blots performed on three isolates of *Metarhizium spp.* with varying virulence for locusts revealed that *M. anisopliae* isolates ME1 and I91 663 produced one major polypeptide of 75 kDa which reacted to the HSP70 antibody. This polypeptide is the same size as the major heat shock inducible HSP70-like polypeptide detected in *M. flavoviride* isolate IMI 330189. In addition all isolates produced a number of smaller isoforms at 27 and 32°C. In all three isolates there was an increase in production of the 75 kDa polypeptide during heat shock at 32 °C (Figure 4-6). At 45°C a decrease in the amount of this polypeptide was detected in *M. anisopliae* isolate I91 633 and *M. flavoviride* isolate IMI 330189 organisms. In addition all of the isolates stopped producing the smaller isoforms of HSP70-like protein.

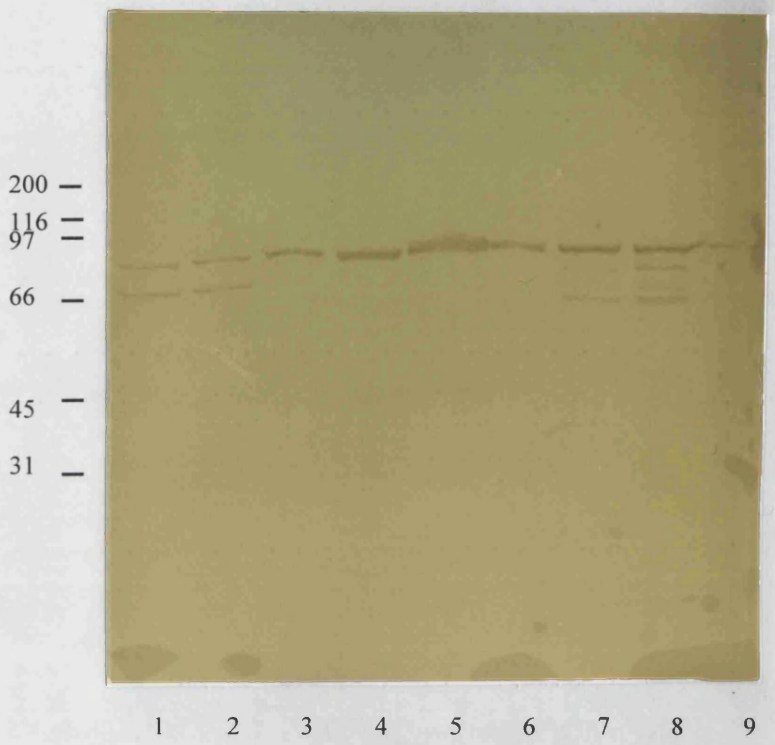
4.3.2.5 Proteins present in *Schistocerca gregaria* haemolymph during infection

After looking at the production of proteins *in vitro* in fungi, the proteins in infected insect haemolymph were studied. The aim of this work was to ascertain whether any of the proteins produced *in vitro* by the fungus might be important *in vivo* during infection.

Only male adult *S. gregaria* were used for these experiments because the haemolymph from different sexes of Acridids is known to vary greatly in both protein content and composition (Miranpuri *et al.*, 1991). The insects were also fed daily because starvation is also known to have an effect on the haemolymph composition.

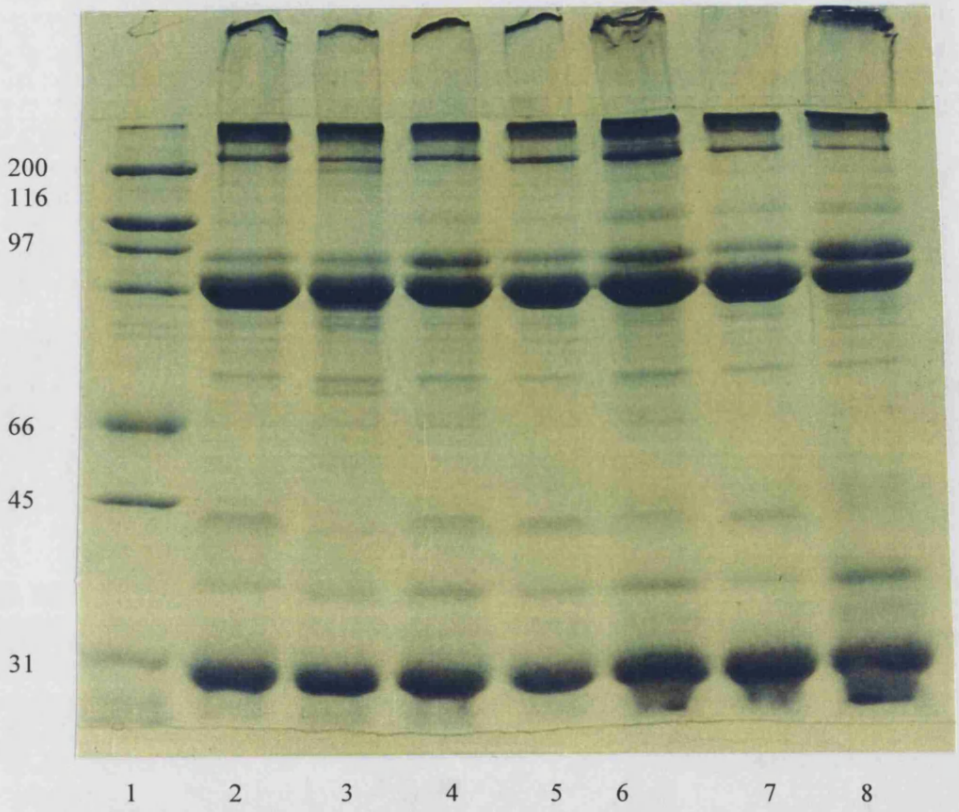
Infected and non-infected insects were bled daily as described in the methods and the proteins from the haemolymph run onto an SDS-PAGE gel (Figure 4-7). The major 74kDa protein detected in the haemolymph is probably the apolipophorin II protein which is responsible for lipid transport in the haemolymph. The 22.5kDa protein is probably an albumin-like protein (Kulkarni and Mehrotra, 1970).

Figure 4-6. Anti-HSP70 probed Western blot of intracellular protein extracts from *Metarhzium anisopliae* isolates ME1 and I91 633 and *Metarhzium flavoviride* isolate IMI 330189 mycelia after heat shock for one hour at 27°C, 32°C or 45°C.



Each of the above lanes was the result of blotting 50 µg of intracellular proteins from *Metarhizium spp* which had been extracted after 1 hours heat shock. Broad range molecular weight marker sizes are indicated on the left in kDa. Lanes 2-10 contain 50 µg of intracellular protein extracted from *M. anisopliae* ME1 (lanes **1-3**) *M. flavoviride* IMI 331089 (lanes **4-6**), and *M. anisopliae* I91 633 (lanes **7-9**). Heat shock conditions were an hour at 27 °C (lanes **1, 4** and **7**); an hour at 32 °C (lanes **2, 5** and **8**) and an hour at 45 °C (lanes **3, 6** and **9**).

Figure 4-7. 10% SDS-PAGE gel with haemolymph proteins extracted from male adult locust infected with 1.5×10^{8} spores of *Metarhizium flavoviride* IMI 330189 in ondina el oil.



Lane **1**, molecular weight marker with sizes in kDa marked on the left hand side; lane **2**, non-infected (inoculated only with oil) haemolymph day 0 (1 hour after inoculation); lane **3**, non-infected haemolymph day 1; lane **4**, infected haemolymph day 1; lane **5**, non-infected haemolymph day 2; lane **6**, infected haemolymph day 2; lane **7**, non-infected haemolymph day 3; lane **8**, infected haemolymph day 3.

Table 4-9. Results from three replicate image analysis scans performed on SDS-PAGE gels of non-infected and infected haemolymph from adult male *Schistocerca gregaria* inoculated with 1.5×10^8 conidia in ondina el oil.

Protein Mr. kDa	Non-infected haemolymph % of total (±s.e.)	Infected haemolymph % of total (±s.e.)			Significant change		
	day 0 - day 3	day1	day2	day 3	day 1	day 2	day 3
173	3.75 (1.08)	2.84 (0.78)	3.61 (1.22)	6.07 (1.82)	-	-	-
158	4.57(0.54)	3.21 (1.33)	4.74 (1.55)	3.95 (1.03)	-	-	-
100	3.83 (0.44)	5.80 (1.12)	5.96 (0.73)	7.03 (0.78)	-	+	+
92	4.45 (1.43)	4.66 (1.26)	3.15 (0.53)	3.61 (0.66)	-	-	-
87	7.14 (2.61)	5.82 (1.46)	5.09 (0.80)	8.69 (2.20)	-	-	-
80.5	5.92(1.09)	4.08 (0.65)	5.06 (0.48)	-	-	-	+
74	1.78(0.39)	2.16 (0.57)	1.97 (0.63)	1.23 (0.03)	-	-	-
69.5	1.10(0.17)	1.03 (0.10)	1.42 (0.36)	1.38 (0.20)	-	-	-
59.5	2.37(0.47)	2.63 (0.28)	1.82 (0.49)	2.08 (0.53)	-	-	-
31	3.14 (0.36)	2.80 (0.53)	3.32 (1.06)	0.88 (0.18)	-	-	-
22.5	2.23(1.32)	3.19 (0.61)	3.37 (1.14)	3.07 (2.34)	-	-	-
19.5	1.19(0.87)	1.45 (0.32)	1.70 (0.71)	2.11 (0.24)	-	-	+
17	1.07(0.21)	1.17 (0.16)	1.14 (0.39)	9.94 (4.47)	-	-	-
15	5.77(0.22)	4.48 (0.83)	7.36 (2.09)	8.62 (1.54)	-	-	-
14	6.10(0.76)	4.50 (0.69)	6.03 (1.68)	2.29 (1.32)	-	-	-
13	3.44(1.47)	4.41 (1.04)	2.18 (0.99)	3.18 (0.24)	-	-	-

Data represent average percentage of total protein from three replicate samples. There were no significant differences in the non-infected haemolymph on different days post-inoculation so these data were pooled. Figures in brackets indicate the standard error. Infected haemolymph was compared to non-infected haemolymph using the student t-test ($\alpha = 0.05$).

Image analysis revealed that the quantity of most of the proteins present did not change significantly during the course of infection. On days two and three of infection there was a significant increase in a 100kDa protein, there was also a significant increase in the quantity of the 19.5kDa polypeptide on day three of infection. Also on day three of infection the 80.5kDa polypeptide disappeared (Table 4-9).

4.3.2.6 ^{35}S labeling of proteins produced in *Schistocerca gregaria* haemolymph on each day of infection

In order to observe specifically those proteins produced on each day during the infection the experiment was repeated labeling the proteins with ^{35}S methionine. This was done by injecting the insects with 5μCi of ^{35}S methionine on each day of infection and extracting the haemolymph after 1 hour of incubation. The total and TCA precipitable counts are both shown in Table 4-10.

It was found that on day 4 of infection the percentage of incorporation of the label increased 10 fold. As would be expected there was no difference in the proteins labeled in samples of control haemolymph each day (Figure 4-8). However there were substantial differences in the pattern of infected haemolymph proteins labeled. On all days of infection there was a decrease in the quantity of labeled protein of 44.7 and 28.2 kDa when compared with the non-infected insect haemolymph. On day two of infection there was an appearance of a 32kDa band, which was not seen in any of the other samples.

The most dramatic change in protein synthesis was seen on the fourth day of infection, the day before death. On this day the incorporation of label was 10 fold greater than for all the other treatments (Table 4-10). Only three bands could be clearly distinguished here; 135, 94.4 and 72 kDa. The most significant bands were those of 94.4 and 72kDa. The 135 and 72kDa band were not observed in any of the other haemolymph samples.

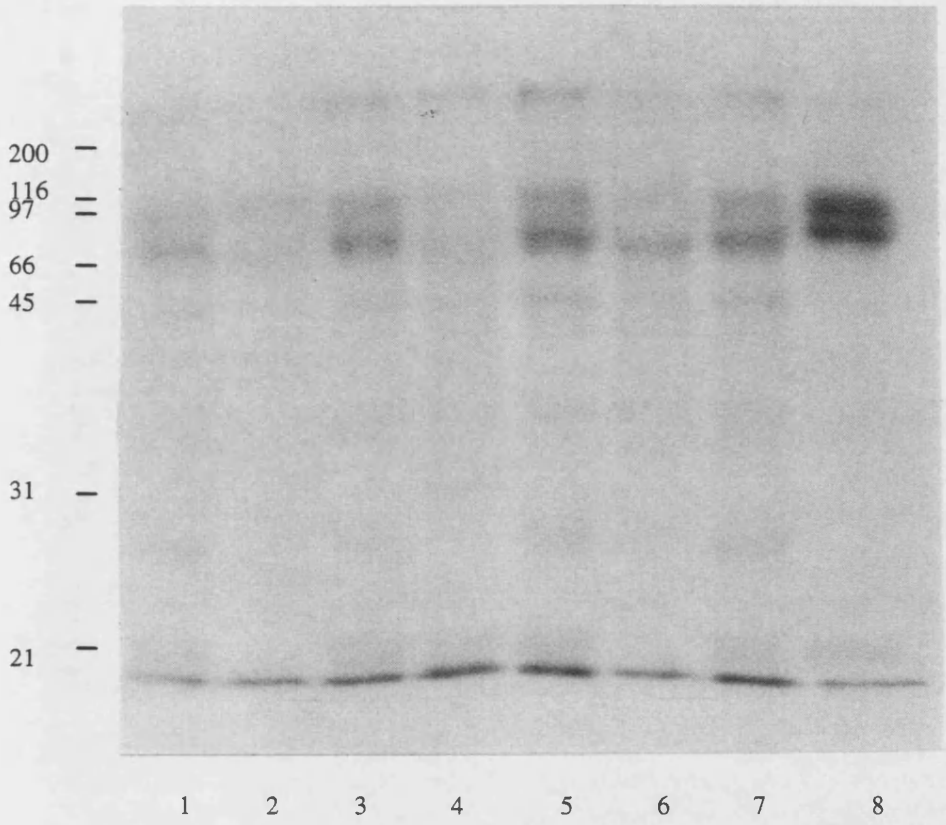
Western blots from control and infected haemolymph from adult male locust (infection at 1.5×10^7 conidia per insect in 5μl of ondina el oil revealed the presence of proteins

antigenically related to HSP70 only in infected blood, in a small quantity on day 2 and in a larger quantity on day 3. No immunogenic material was detected in the infected haemolymph on day 4 despite the fact that there was a large increase in protein synthesis on this day. The amount of binding of antibody in the haemolymph was very small compared to the amount obtained from intracellular protein extract from *M. flavoviride* and the blots had to be developed for at least one hour as opposed to the 5 minutes taken to develop *M. flavoviride in vitro* blots.

Table 4-10. Incorporation of ^{35}S methionine in infected and non-infected adult male *Schistocerca gregaria* haemolymph *in vivo*.

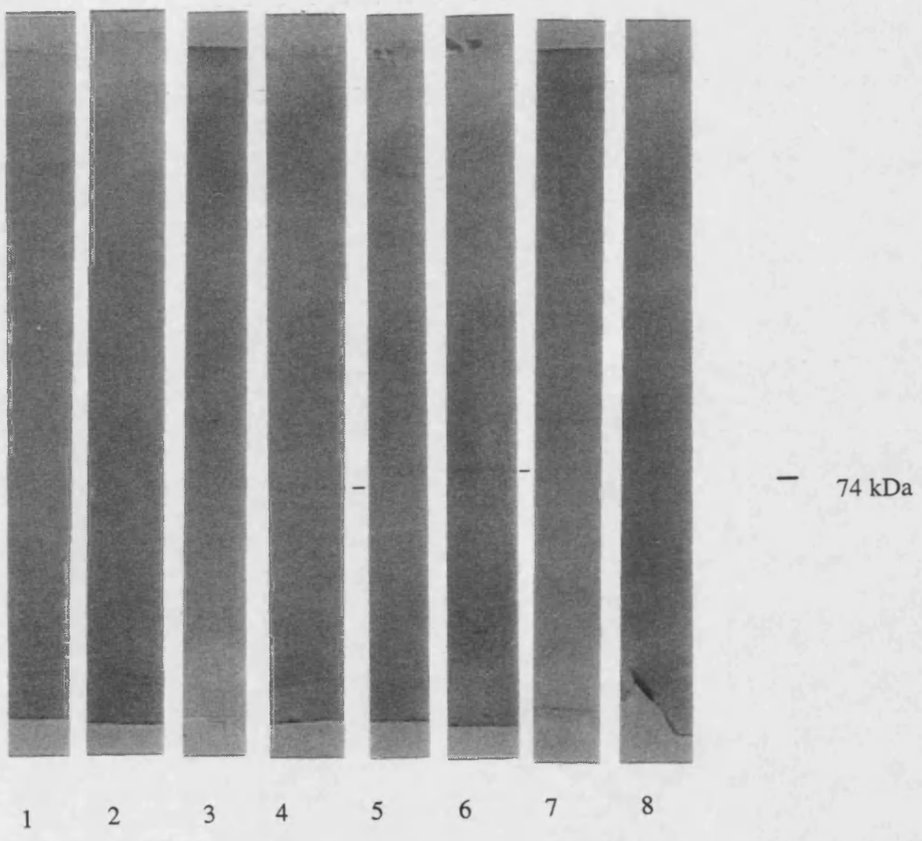
Sample	dpm/μl		% incorporation
	Total	TCA precipitable	
Control, day 1	83972.5	3316.2	3.95
Control, day 2	30291.8	2192.5	7.24
Control, day 3	51587.5	2314.9	4.89
Control, day 4	61285.4	5799.4	9.46
Infected, day 1	44251.4	1458.4	3.30
Infected, day 2	39422.0	1390.2	3.53
Infected, day 3	75677.5	4040.4	5.34
Infected, day 4	78856.0	27748.5	35.19

Figure 4-8. ^{35}S-methionine labeled haemolymph proteins extracted from male adult locust infected with 1.5×10^{7} spores of *Metarhizium flavoviride* IMI 330189 in ondina el oil.



The position and size (in kDa) of the molecular weight markers are indicated on the left. Lanes 1-7 contain 2000 dpm TCA precipitable counts. Lane **1**, non-infected (inoculated only with oil) haemolymph day 1; lane 2, infected haemolymph day 1; lane **3**, non-infected haemolymph day 2; lane **4**, infected haemolymph day 2; lane **5**, non-infected haemolymph day 3; lane **6**, infected haemolymph day 3; lane **7**, non-infected haemolymph day 4; lane **8**, infected haemolymph day 4.

Figure 4-9. Anti-HSP70 probed Western blot of haemolymph proteins extracted from male adult locust infected with 1.5×10^7 spores of *Metarhizium flavoviride* isolate IMI 330189 in ondina el oil.



Lane **1**, non-infected haemolymph day 1; lane **2**, infected haemolymph day 1; lane **3**, non-infected haemolymph day 2; lane **4**, infected haemolymph day 2; lane **5**, non-infected haemolymph day 3; lane **6**, infected haemolymph day 3, lane **7**, non-infected haemolymph day 4; lane **8**, infected haemolymph day 4.

4.3.3 Molecular studies of *hsp70* and *hsp90*

In order to study the heat shock proteins of *Metarhizium spp.* at the molecular level it was decided to amplify the relevant areas of genomic DNA using the polymerase chain reaction (PCR). This was a technique especially suitable for heat shock proteins genes due to their highly conserved nature. Two genes were investigated, *hsp70* and *hsp90*.

4.3.3.1 Primer design

Degenerate oligonucleotide primers were designed to homologous regions from alignments of deduced amino acid sequences from a number of fungal and yeast heat shock genes from the EMBL database for both *hsp70* (Table 4-11) and *hsp90* (Table 4-12).

Table 4-11. An alignment of homologous areas from the deduced amino acid sequence of the *hsp70* gene from three organisms from which the *hsp70* oligonucleotide primers were designed.

Species	Accession no.	*hsp70-1*	*hsp70-2*	*hsp70-3*	*hsp70-4*
Achyla klebsiana	Em_fun:Ak02504	IIANDQGN	IGDAAKN	INPDEAVA	GAAVQGA
Blastocladiella emersonii	Em_fun:Bsiahsp70	IIANDQGN	IGDAAKN	INPDEAVA	GAAVQGA
Saccharomyces cerivisiae	Em_fun:Schsp70a	IIANDQGN	IGDAAKN	INPDEAVA	GAAVQGA

Table 4-12. An alignment of homologous areas from the deduced amino acid sequence of the *hsp90* gene from two organisms from which the *hsp90* oligonucleotide primers were designed.

Species	Accession no.	*hsp90-1*	*hsp90-2*	*hsp90-3*
Schizosaccharomyces pombe	Gb_pl:Ysphsp90x	KKNNIK	FNEIAE	EETDEEK
Saccharomyces cerivisiae	Em_fun:Schsp90	KKNNIK	FNEIAE	EETDEEK

Four different oligonucleotide primers were designed for the amplification of *hsp70* genes in *Metarhizium spp.*, two to read in a 5' to 3' direction and two to read in a 3' to 5' direction. The inner pair of oligonucleotides were designed so as to have restriction sites at either end (Figure 4-10). This had two advantages, firstly it allowed the use of a method known as nested PCR. This involves an initial amplification round with the outer pair of primers (*hsp70-1* and *hsp70-4*) followed by a second round of amplification using the PCR products produced in the first round as template with the inner pair of primers (*hsp70-2* and *hsp70-3*). This leads to a very pure product which is more likely to be part of the gene of interest. The second advantage of these oligonucleotides is that the inner primers contain restriction sites which makes cloning the products a much simpler process. Note that there are additional nucleotides added before the restriction site as this has been found to be necessary for efficient restriction. The position of these primers in relation to a typical *hsp70* gene consensus sequence is shown in Figure 4-11.

Figure 4-10. Oligonucleotide sequence of primers designed for PCR amplification of *hsp70* sequence from genomic *Metarhizium anisopliae* and *Metarhizium flavoviride* DNA.

hsp70-1	**IIANDQGN** ⇒		
5'	ATC/T ATC/T GCC/T AAC/T gAC/T CAA/g gGC/T AA	3'	23 mer
hsp70-2	**IGDAAKN** ⇒		
5'	AA gAA TTC ggC/T gAC/T gCC/T gCC/T AAA/g AAC/T CA	3'	20 mer
hsp70-3	**INPDEAVA** ⇐		
5'	AA ggA TCC A/ggC A/gAC A/gAC A/ggC C/TTC A/gTC A/ggg A/gTT	3'	24 mer
hsp70-4	**GAAVQAA** ⇐		
5'	AT Agg/C A/ggC C/TTg A/gAC A/ggC A/ggC	3'	20 mer

The primer name is given in italics, the amino acid sequence to which it was designed is given in bold and the nucleotide sequence is given below. Underlined regions represent restriction sites for EcoRI in oligonucleotide primer *hsp70-2* and for BamHI in *hsp70-3*. / between two nucleotides indicates that the sequence was degenerate at this point and may have contained either of the two nucleic acids. ⇒ represents a forward reading primer and ⇐ a reverse reading primer. The number of nucleotides in the primer are indicated on the right (N.B. this number does not include the nucleotides contained in the restriction sites).

Figure 4-11. A diagrammatic representation of the positions of the four *hsp70* oligonucleotide primers and their restriction sites in relation to a typical *hsp70* gene consensus sequence.

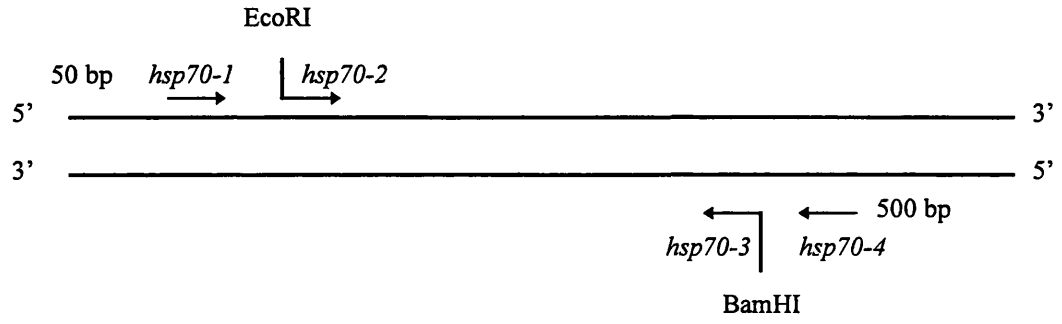


Diagram not to scale. Estimated total gene length ~ 2 kb, estimated PCR fragment sizes all ~ 1.1 kb.

Three oligonucleotide primers were also designed for the amplification of *hsp90*, two to read in a 5' to 3' direction and one to read in a 3' to 5' direction. The sequence of these primers is given in Figure 4-12 and their positions within a typical *hsp90* consensus gene are illustrated in Figure 4-13.

Figure 4-12. Oligonucleotide sequence of primers designed for PCR amplification of *hsp90* sequence from genomic *Metarhizium anisopliae* and *Metarhizium flavoviride* DNA.

hsp90-1 **KKNIK** ⇒

5'	AA gAA TTC AAA/g AAA/g AAT/C AAT/C AAA/T/C AA	3'	17 mer

hsp90-2 **FFNEIA** ⇒

5'	AA gAA TTC TTT/C AAT/C gAg/A ATA/T/C gCN gA	3'	17 mer

hsp90-3 **EETDEEK** ⇐

5'	AA ggA TCC T/CTT T/CTC T/CTC A/gTC NGT T/CTC T/CTC	3'	21 mer

The primer name is given in italics, the amino acid sequence to which it was designed is in bold and the nucleotide sequence is given below. Underlined regions represent restriction sites for EcoRI in oligonucleotide *hsp90-1* and *hsp90-2* and for BamHI in *hsp90-3*. / between two nucleotides indicates that the sequence was degenerate at this point and may have contained either of the two nucleic acids. ⇒ represents a forward reading primer and ⇐ a reverse reading primer. The number of nucleotides in the primer are indicated on the right (N.B. this number does not include the restriction sites).

Figure 4-13. A diagrammatic representation of the positions of the three *hsp90* oligonucleotide primers and their restriction sites in relation to a typical *hsp90* gene consensus sequence.

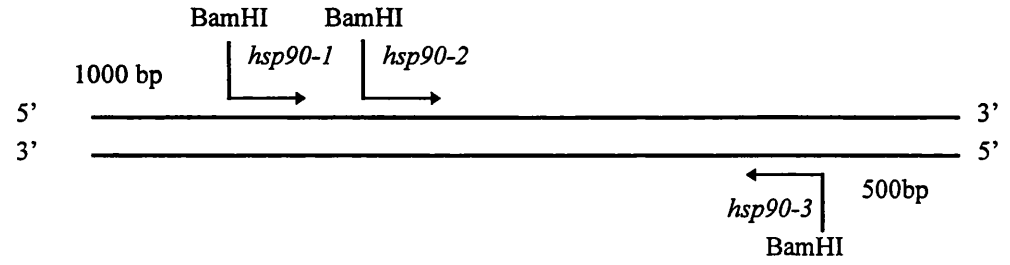


Diagram not to scale. Expected gene size ~ 2.1 kb, expected fragment sizes *hsp90-1* to *hsp90*-3 ~ 400 kb and *hsp90-2* to *hsp90-3* ~ 600 kb.

The PCR conditions used for the amplification of both *hsp70* and *hsp90* are listed in the materials and methods.

4.3.3.2 Amplification of *hsp70* from *Metarhizium anisopliae* isolate ME1 using the PCR.

Initial amplification from *M. anisopliae* isolate ME1 genomic DNA with the *hsp70* primers gave single bands between primers *hsp70-1* + *hsp70-3* and *hsp70-2* + *hsp70-3* of about 1.3 kb. Many fragments were produced between primers *hsp70-1* + *hsp70-4* and *hsp70-2* + *hsp70-4* but both reactions also contained bands of approximately 1.3 kb (Figure 4-14). This 1.3 kb product was slightly larger than the expected 1.1 kb fragment predicted from the consensus sequence shown in Figure 4-11.

A Southern blot of the PCR fragments generated between primers *hsp70-1* + *hsp70-3* and *hsp70-1* + *hsp70-4* was screened with radioactively labeled oligonucleotide primer *hsp70-2*. The single band produced between oligonucleotides *hsp70-1* and *hsp70-3* lit up as well as one of the multiple bands produced between *hsp70-1* and *hsp70-4*. Both

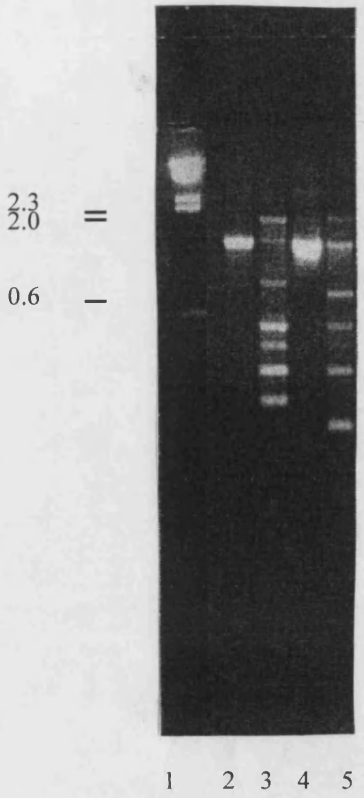
these bands were approximately 1.3 kb in size. The strong signal at the bottom of the Southern blot was probably due to excess primer *hsp70-2* that had diffused across from the adjacent lane (Figure 4-15). Nested PCR was then used to improve the purity of the products and to introduce restriction sites into the fragment. This was achieved by using the product amplified between primers *hsp70-1* and *hsp70-4* as a template in a further PCR using primers *hsp70-2* and *hsp70-3*. This again gave rise to a single PCR fragment of approximately 1.3 kb which was then known to have arisen from DNA which had sequence homology with all four *hsp70* oligonucleotides.

4.3.3.3 Cloning and sequencing the *hsp70* PCR product from *Metarhizium anisopliae* isolate ME1

The nested PCR fragment produced using the *hsp70* oligonucleotide primers was cloned and double strand sequenced as described in the methods in order to ascertain whether this was a fragment of the *M. anisopliae hsp70* gene (Figure 4-16). The sequence obtained from this clone showed significant homology to other *hsp70* genes and is shown in Figure 4-17. This fragment represents about 50% of the sequence encoding the *hsp70* gene.

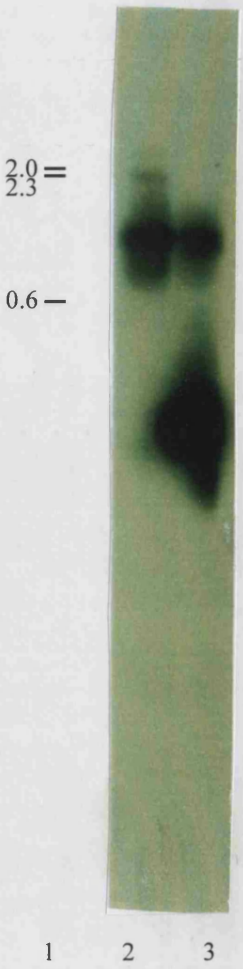
It appears from sequence data that this PCR product contains an intron, this explains why the product amplified was larger than expected. Evidence for the presence of the intron comes from the fact that the sequence alignment is interrupted by an area which contains a number of stop codons and consensus sequences typical of intron splice junctions from filamentous fungi. A typical consensus sequence for the 5' splice site of an intron is GTANGT, in *M. anisopliae*'s *hsp70* the sequence at the proposed start of the intron is GTAAGA. The 3' end of the intron is always marked by AG usually preceded by a pyrimidine as it is in this putative *M. anisopliae* intron. In addition a sequence closely matching the consensus sequence for lariat formation occurs 6 bp upstream from the 3' splice site. The 117 bp sequence is also the right size for a fungal intron, which range between 48 and 323bp (Gurr *et al.*, 1987).

Figure 4-14. Products amplified by the polymerase chain reaction from genomic *Metarhizium anisopliae* isolate ME1 DNA using the *hsp70* oligonucleotides.



PCR conditions are listed in methods. Lane **1**, HindIII restricted λ marker with sizes marked in kb on the left; PCR products produced between oligonucleotides lane **2**, *hsp70-1* and *hsp70-3*; lane **3**, *hsp70-1* and *hsp70-4*; lane **4**, *hsp70-2* and *hsp70-3*; lane **5**, *hsp70-2* and *hsp70-4*, lane **6**, nested PCR product; lane **7**, control reaction with no DNA; lane **8**, cleaned nested PCR product.

Figure 4-15. Screening of a Southern blot of the *hsp70* PCR products with ^{32}P labeled internal oligonucleotide *hsp70-2*.



Lane **1**, HindIII restricted λ marker with sizes marked in kb; lane **2**, PCR products formed between oligonucleotides *hsp70-1* and *hsp70-3*; lane **3**, PCR products formed between oligonucleotides *hsp70-1* and *hsp70-4*.

Introns are reported to be present in about 63% of fungal genes (although evidence for this is mostly based on sequence information only). The position (but not sequence) of intron sites in genes tend to be conserved between fungi and even to some degree with higher eukaryotes. In *M. anisopliae* the intron is nine nucleotide base pairs further downstream than one of the four *Neurospora crassa hsp70* introns. However it did not contain another two introns which were present in this part of the *Neurospora crassa* gene (Kapoor *et al.*, 1995). Comparisons of intron position with other *hsp70* genes from humans (Hunt and Morimoto, 1985), sea urchins (Rosa *et al.*, 1990), worms (Snutch *et al.*, 1988), Hydra (Gellner *et al.*, 1992), maize (Rochester *et al.*, 1986) and *Blastocladiella emersonii* (Stefani and Gomes, 1995) did not reveal any other similarities.

An alignment of the sequence obtained from the *M. anisopliae* PCR product showed that the most closely related gene on the database was the *hsp70* gene from *Neurospora crassa* which showed 82.0% identity to the deduced amino acid sequence of *M. anisopliae*'s HSP70 (Table 4-13). Alignments of the *M. anisopliae hsp70* clone with a number of other deduced amino acid sequences are given in Figure 4-17. This amino acid alignment revealed some interesting differences between *M. anisopliae* and all other organisms. The most striking difference was that *M. anisopliae* contained at least 6 extra amino acids between positions 247 and 253 in the line up (excluding the virus sequence). Comparisons of *M. anisopliae* with the 40 most closely matching sequences in the database revealed that these extra bases were not present in any other organisms despite their wide range of origins (fungi, bacteria, plants and animals). However there were variations in the size of this gap which does indicate that it is a more variable area of the protein. There were also a number of positions in which *M. anisopliae* showed one amino acid difference with all other organisms (244) or all other fungi (279, 361, 363, 394 and 398). These differences could be due to errors introduced during amplification with Taq and in order to check the sequence it will be necessary to clone the genomic DNA from the λ library (this work is presently being carried out). It is also

interesting to note that this fragment of *M. anisopliae*'s HSP70 shows higher homology to the pig (*Sus scrofa*) HSP70 than some of the other fungi.

4.3.3.4 Copy number of *hsp70* within *Metarhizium anisopliae* isolate ME1

Screening of a Southern blot of a digested genomic DNA from *M. anisopliae* isolate ME1 with the cloned *hsp70* PCR product revealed the presence of only one band with homology to the cloned product in all cases. This gives a strong indication that *hsp70* in *M. anisopliae* is a single copy gene.

Figure 4-16. DNA sequence obtained from PCR clone of *hsp70* amplified by nested PCR from *Metarhizium anisopliae* ME1.

```
GAATTCGGTGATGCTGCTAAAAATCAGGTCACCAAGAACCCTGAGAACACAGTCTTTGAC      34
                          V  T  K  N  P  E  N  T  V  F  D         11

GCCAAGCGATTGATTGGCCGAAAGTTCTCCGATGCCGATGTCCAGGCCGACATGAAGCAC      94
A  K  R  L  I  G  R  K  F  S  D  A  D  V  Q  A  D  M  K  H       31

TTCCCCTTCAAGGTCATCGATAAGTCCGGCAAGCCCGCTGTCGAGGTCCAATACATGGGC      154
F  P  F  K  V  I  D  K  S  G  K  P  A  V  E  V  Q  Y  M  G       51

GAGACCAAGACCTTCACACCTGAGGAAATCTCATCTATGATTCTCATGAAGATGCGTGAG      214
E  T  K  T  F  T  P  E  E  I  S  S  M  I  L  M  K  M  R  E       71

ACCGCAGAGGCTTTTATTGGCGAGACTGTCAACAATGCCGTCATCACTGTCCCTGCCTAC      274
T  A  E  A  F  I  G  E  T  V  N  N  A  V  I  T  V  P  A  Y       91

TTCAACGACTCTCAGCGTCAGGCCACCAAGGACGCTGGTCTCATCGCCGGCCTGAACGTC      334
F  N  D  S  Q  R  Q  A  T  K  D  A  G  L  I  A  G  L  N  V       111

CTGCGAATCATCAACGAACCCACTGCCGCCGCCATTGCCTATGGCTTCGACAAGATTCGC      394
L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G  F  D  K  I  R       131

CCTGAGGACAAGGACAAGGATGATATCCCCGAGCGCAATATCCTCATCTTCGATCTTGGT      454
P  E  D  K  D  K  D  D  I  P  E  R  N  I  L  I  F  D  L  G       151

GGTGGTACTTTCGATGTCTCCCTCCTCGCCGTTGCCGGAGGTGTCTTTGAAGTCAAGTCC      514
G  G  T  F  D  V  S  L  L  A  V  A  G  G  V  F  E  V  K  S       171

ACCGCTGGTGACACTCACTTGGGTGGTGAGGACTTTGACAACCGATTGGTGAACCACTTT      574
T  A  G  D  T  H  L  G  G  E  D  F  D  N  R  L  V  N  H  F       191

GTAAATGAGTTCAAACGCAAGCACAAGAAGGgtaagagcgctcttttccaatataattga      634
V  N  E  F  K  R  K  H  K  K                                      201

gcatttttgagtacatttggatgtattcgaatatgctcgagtatatctcagtacagtcaa      694
                                                                  201


gatgaacatggagctaacttgataacagATCTCACCACCAACTCACGTGCTCTGCGCCGT      754
                             L  T  T  N  S  R  A  L  R  R         211

CTTCGAAGCGCCTGTGAGCGTGCCAAGCGTGCCCTGTCTTCGGCTTCCCAGACCTCTATT      814
L  R  S  A  C  E  R  A  K  R  A  L  S  S  A  S  Q  T  S  I       231
```

```
GAGATCGACTCTCTGATGGAGAACATCGACTTCTACACCTCCATCACCCGAGCTCGTTTC      874
E  I  D  S  L  M  E  N  I  D  F  Y  T  S  I  T  R  A  R  F       251

GAAGAGCTTTGCCAGGATCTCTTCCGTTCTACCATGCAGCCTCTCGACCGCGTCATCTCG      934
E  E  L  C  Q  D  L  F  R  S  T  M  Q  P  L  D  R  V  I  S       271

GATGCCAAGATCGACAAGAACCAGGTTGACGAGATTGTCCTCGTCGGTGGCTCTACCCGT      994
D  A  K  I  D  K  N  Q  V  D  E  I  V  L  V  G  G  S  T  R       291

ATCCCCCGTATCCAGAAGCTCATCACTGAGTACTTCAACGGCAAGCAGCCCAGCAAGTCC      1054
I  P  R  I  Q  K  L  I  T  E  Y  F  N  G  K  Q  P  S  K  S       311

ATCAACCCCGACGAAGCCGTTGCCGGATCC                                    1057
I                                                                 312
```

Underlined regions of the sequence represent restriction sites for EcoRI and BamHI. Bases in bold uppercase represent the oligonucleotide primers used to amplify the sequence. All letters in lowercase represent the proposed position of an intron, with bases in lowercase bold defining the position of the intron. The 5' start site of the intron is defined by the consensus sequence GTANGT, the internal consensus sequence for lariat formation is PyGCTAAC and the 3' consensus sequence marking the end of the intron is PyAG, where Py = pyrimidine (Gurr *et al.*, 1987).

Figure 4-17. An alignment of the derived amino acid sequences of *hsp70* from the PCR clone from *Metarhizium anisopliae* isolate ME1 and a number of other organisms.

```
         1                                                  50
    Ma  .......... .......... .......... .......... ..........
    Nc  .......... .......... .......... .......... ..........
    Pg  .......... .......... .......... .......... ..........
  Ssa1  .......... .......... .......... .......... ..........
    Ca  .......... .......... .......... .......... ..........
  Ssa2  .......... .......... .......... .......... ..........
    Be  .......... .......... .......... .......... ......MTTK
    Dd  .......... .......... .......... .......... ..........
    Ss  .......... .......... .......... .......... .........M
    Ak  .......... .......... .......... .......... .......MSG
    Km  .......... .......... .......... .......... .......AEG
    Sp  .......... .........MK KFQLFSILSY FVALFLLPMA FASGDDNSTE
    Hs  MISASRAAAA RLVGAAASRG PTAARHQDSW NGLSHEAFRL VSRRDYASEA
    Ec  .......... .......... .......... .......... ..........
sbyvir  .......... .......... .......... .......... ..........

         51                                                 100
    Ma  .......... .......... .......... .......... ..........
    Nc  .MAPAVGIDL GTTYSCVGVF REDRCEIIAN DQGNRTTPSF VAFT.DTER.
    Pg  .MTKAIGIDL GTTYSCVGVW QNDRVEIIAN DQGNRTTPSY VAFT.DTER.
  Ssa1  ..SKAVGIDL GTTYSCVAHF ANDRVDIIAN DQGNRTTPSF VAFT.DTER.
    Ca  ..SKAVGIDL GTTYSCVAHF ANDRVEIIAN DQGNRTTPSF VAFT.DTER.
  Ssa2  ..SKAVGIDL GTTYSCVAHF SNDRVDIIAN DQGNRTTPSF VGFT.DTER.
    Be  LDSPAVGIDL GTTYSCVGVW QNDRVEIIAN DQGNRTTPSY VGFT.DSER.
    Dd  ..MSSIGIDL GTTYSCVGVW QNDRVEIIAN DQGNRTTPSY VAFT.DTER.
    Ss  AKSVAIGIDL GTTYSCVGVF QHGKVEIIAN DQGNRTTPSS VAFT.DTER.
    Ak  VQGASVGIDL GTTYSCVGVW QNDRVEIIAN DQGNRTTPSY VAFT.DSER.
    Km  VFPGAIGIDL GTTYSCVATY ENS.VEIIAN EQGNRVTPSF VAFT.PEER.
    Sp  SYGTVIGIDL GTTYSCVAVM KNGRVEIIAN DQGNRITPSY VAFT.EDER.
    Hs  IKGAVVGIDL GTTNSCVAVM EGKQAKVLEN AEGARTTPSV VAFTADGER.
    Ec  ..GKIIGIDL GTTNSCVAIM DGTTPRVLEN AEGDRTTPSI IAYTQDGET.
sbyvir  ..MVVFGLDF GTTFSSVCAY VGEELYLF.K QRDSAYIPTY VFLHSDTQEV

         101                                                150
    Ma  .EFGDAAKNQ VTKNPENTVF .DAKRLIGRK FSD.....AD VQADMKHFPF
    Nc  .LVGDAAKNQ VAMNPANTVF .DAKRLIGRK FSD.....PE VQADMKHFPF
    Pg  .LIGDSAKNQ VAMNPHNTVF .DAKRLIGRK FAD.....AE VQSDIKHFPF
  Ssa1  .LIGDAAKNQ AAMNPSNTVF .DAKRLIGRN FND.....PE VQADMKHFPF
    Ca  .LIGDAAKNQ AAMNPANTVF .DAKRLIGRK FDD.....PE VINDAKHFPF
  Ssa2  .LIGDAAKNQ AAMNPANTVF .DAKRLIGRN FND.....PE VQGDMKHFPF
    Be  .LIGDAAKNQ VAMNPHNTVF .DAKRLIGRR FDD.....DV VQADMKHWSF
    Dd  .LIGDAAKNQ VAMNPTNTVF .DAKRLIGRK FSD.....KE VQSDMKHWPF
    Ss  .LIGDAAKNQ VALNPQNTVF .DAKRLIGRK FGD.....PV VQGDMKHWPF
    Ak  .LIGDAAKNQ VAMNPANTVF .DAKRLIGRK FND.....PA TQADIKHWPF
    Km  .LIGDAAKNQ AALNPKNTVF .DAKRLIGRR FDE.....ES VQSDMKTWPF
    Sp  .LVGEAAKNQ APSNPENTIF .DIKRLIGRK FDE.....KT MAKDIKSFPF
    Hs  .LVGMPAKRQ AVTNPNNTFY .ATKRLIGRR YDD.....PE VQKDIKNVPF
    Ec  .LVGQPAKRQ AVTNPQNTLF .AIKRLIGRR FQD.....EE VQRDVSIMPF
sbyvir  AFGYDAEVLS NDLSVRGGFY RDLKRWIGCD EENYRDYLEK LKPHYKTELL
```

```
         151                                                  200
    Ma   KVIDKSG.KP AVEVQYMGET KTFTPEEISS MILMKMRETA EAFIGETVNN
    Nc   KVIDRGG.KP VIQVEFKGET KVFTPEEISA MILQKMKETA EAYLGGTVNN
    Pg   TVFDKGG.KP YIRVQYRGED KEFSPEEISS MILTKMKEVA ESYLGGTVTS
  Ssa1   KLIDVDG.KP QIQVEFKGET KNFTPEQISS MVLGKMKETA ESYLGAKVND
    Ca   KVIDKAG.KP VIQVEYKGET KTFSPEEISS MVLTKMKEIA EGYLGSTVKD
  Ssa2   KLIDVDG.KP QIQVEFKGET KNFTPEQISS MVLGKMKETA ESYLGAKVND
    Be   TVVNKNS.KP LFQVEFKGET KTFTPEEFSS MILTKMKETA EAYLGTKVNH
    Dd   KVIPKDGDKP HIQVEFKGET KVFSPEEISS MVLLKMKETA EAYLGKTINN
    Ss   RVINDGD.KP KVQVSYKGET KGFYPEEISS MVLTKMKEIA EGYLGHPVSN
    Ak   KVTPGAGDKP QITVEFKGET KTFQPEEISS MVLIKMKEVA EAFIGTAVNN
    Km   KVIDSNGA.P LIEVEYLGET KTFSPQEISS MVLTKMKEIA EAKIGKKVEK
    Sp   HIVN.DKNRP LVEVNVGGKK KKFTPEEISA MILSKMKQTA EAYLGKPVTH
    Hs   KIVRASNGDA WVEA....HG KLYSPSQIGA FVLMKMKETA ENYLGHTAKN
    Ec   KIIAADNGDA WVEV....KG QKMAPPQISA EVLKKMKKTA EDYLGEPVTE
sbyvir   KVAQSSKSTV KLDCYSGTVP QNATLPGLIA TFVKALISTA SEAFKCQCTG

         201                                                  250
    Ma   AVITVPAYFN DSQRQATKDA GLIAGLNVLR IINEPTAAAI AYGFDKIRPE
    Nc   AVVTVPAYFN DSQRQATKDA GLIAGLNVLR IINEPTAAAI AYGLDK....
    Pg   AVVTVPAYFN DSQRQATKDA GTISGLNVLR IINEPTAAAI AYGLDK....
  Ssa1   AVVTVPAYFN DSQRQATKDA GTIAGLNVLR IINEPTAAAI AYGLDK....
    Ca   AVVTVPAYFN DSQRQATKDA GTIAGLNVLR IINEPTAAAI AYGLDK....
  Ssa2   AVVTVPAYFN DSQRQATKDA GTIAGLNVLR IINEPTAAAI AYGLDK....
    Be   AVVTVPAYFN DSQRQATKDA GAIAGLNVLR IINEPTAAAI AYGLDK....
    Dd   AVITVPAYFN DSQRQATKDA GTISKLNVQR IINEPTAAAI RYGLE.....
    Ss   AVITVPAYFN DSQRQATKDA GVIAGLNVLR IINEPTAAAI AYGLD.....
    Ak   AVITVPAYFN DSQRQATKDA GAIAGLNVLR IINEPTRAAI AYGLDK....
    Km   AVVTVPAYFN DAQRQATKDA GAIAGLNVLR IINEPTAAAI AYGVGAG...
    Sp   SVVTVPAYFN DAQRQATKDA GTIAGLNVIR IVNEPTAAAI AYGLDK....
    Hs   AVITVPAYFN DSQRQATKDA GQISGLNVLR VINEPTAAAL AYGLDK....
    Ec   AVITVPAYFN DAQRQATKDA GRIAGLEVKR IINEPTAAAL AYGLDK....
sbyvir   VICSVPANYN CLQRSFTESC VNLSGYPCVY MVNEPSAAAL S.........

         251                                                  300
    Ma   DKDKDDIPER NILIFDLGGG TFDVSLLAVA GG....VFEV KSTAGDTHLG
    Nc   ...KVE.GER NVLIFDLGGG TFDVSLLTIE EG....IFEV KSTAGDTHLG
    Pg   ...KT.VGER NVLIFDLGGG TFDVSLLTIE EG....IFEV KATAGDTHLG
  Ssa1   ...KGK..EE HVLIFDLGGG TFDVSLLSIE DG....IFEV KATAGDTHLG
    Ca   ...KGSRGEH NVLIFDLGGG TFDVSLLAID EG....IFEV KATAGDTHLG
  Ssa2   ...KGK..EE HVLIFDLGGG TFDVSLLSIE DG....IFEV KATAGDTHLG
    Be   ...KAEAGEK NVLIFDLGGG TFDVSLLTIE DG....IFEV KATAGDTHLG
    Dd   ...KKGSGEK NILIFDLGGG TFDVSLLTIE DG....VFEV KATAGDTHLG
    Ss   ...RTGKGER NVLIFDLGGG TFDVSILTID DG....IFEV KATAGDTHLG
    Ak   .....KGGER NVLIFDLGGG TFDVSLLTIE EG....IFEV KATAGDTHLG
    Km   ....NSEKER HVLIFDLGGG TFDVSLLHIA GG....VYTV KSTSGNTHLG
    Sp   .....TDTEK HIVVYDLGGG TFDVSLLSID NG....VFEV LATSGDTHLG
    Hs   .....S.EDK VIAVYDLGGG TFDISILEIQ KG....VFEV KSTNGDTFLG
    Ec   .....GTGNR TIAVYDLGGG TFDISIIEID EVDGEKTFEV LATNGDTHLG
sbyvir   ACSRIKGATS PVLVYDFGGG TFDVSVISAL NN....TFVV RASGGDMNLG
```

```
        301                                                  350
    Ma  GEDFDNRLVN HFVN..EFKR KHKKDLTTNS RALRRLRSAC ERAKRALSSA
    Nc  GEDFDNRLVN HFVQ..EFKR KDKKDLSTNA RALRRLRTAC ERAKRTLSSS
    Pg  GEDFDNRLVN HFVQ..EFKR KHKKDLSSNP RALRRLRTAC ERAKRTLSSA
  Ssa1  GEDFDNRLVN HFIQ..EFKR KNKKDLSTNQ RALRRLRTAC ERAKRTLSSS
    Ca  GEDFDNRLVN FFIQ..EFKR KNKKDISTNQ RALRRLRTAC ERAKRTLSSS
  Ssa2  GEDFDNRLVN HFIQ..EFKR KNKKDLSTNQ RALRRLRTAC ERAKRTLSSS
    Be  AEDFDNRLVN HFVQ..EFKR KHKKDLSGKA RP.RRLRTAC ERAKRTLSSS
    Dd  GEDFDNRLVS HFVD..EFKR KHKKDIMGNQ RAVRRLRTAC ERAKRTLSSS
    Ss  GEDFDNRLVN HFVE..EFKR KHKKDYSQNK RAVRRLRTAC ERAKRTLSSS
    Ak  GEDFDNRLVD HFTA..EFKR KHRKDMTQNQ RALRRLRTAC ERAKRTLSSS
    Km  GQDFDTNLLE HFKT..EFKK KTGADISGDA RALRRLRTAA ERAKRTLSSV
    Sp  GEDFDNRVIN YLAR..TYNR KNNVDVTKDL KAMGKLKREV EKANGTLSSQ
    Hs  GEDFDQALLR HIVK..EFKR ETGVDLTKDN MALQRVREAA EKAKCELSSS
    Ec  GEDFDSRLIN YLVE..EFKK DQGIDLRNDP LAMQRLKEAA EKAKIELSSA
 sbvir  GRDIDKAFVE HLYNKAQLPV NYKIDISFLK ESLSK..... KVSFLNFPVV

        351                                                  400
    Ma  SQTSIEIDSL MENI....DF YTSITRARFE ELCQDLFRST MQPLDRVISD
    Nc  AQTSIEIDSL FEGI....DF YTSITRARFE ELCQDLFRST LQPVDRVLTD
    Pg  AQTTIEIDSL FEGV....DF YTSLTRARFE ELCQDLFRST LEPVEKVLRD
  Ssa1  AQTSVEIDSL FEGI....DF YTSITRARFE ELCADLFRST LDPVEKVLRD
    Ca  AQTSIEIDSL YEGI....DF YTSITRARFE ELCADLFRST LDPVGKVLAD
  Ssa2  AQTSVEIDSL FEGI....DF YTSITRARFE ELCADLFRST LDPVEKVLRD
    Be  AQTSIEIDSL FEGI....DF YTSITRARFE ELCADLFRST LDPVEKVLRD
    Dd  AQASIEIDSL FEGI....DF YTSITRARFE ELCADLFRGC LDPVEKVLKD
    Ss  TQASLEIDSL FEGI....DF YTSITRARFE ELCSDLFRST LEPVEKALRD
    Ak  AQAYIEIDSL FDGI....DF NSTITRARFE DMCGDYFRKT MEPVEKVLRD
    Km  AQTTVEVDSL FDGE....DF EATITRARFE DINAALFKST LEPVEQVLKD
    Sp  KSVRIEIESF FNGQ....DF SETLSRAKFE EIKHGSLQED FEPVEQVLKD
    Hs  VQTDINLPYL TMDSSGPKHL NMKLTRAQFE GIVTDLIRRT IAPCQKAMQD
    Ec  QQTDVNLPYI TADATGPKHM NIKVTRAKLE SLVEDLVNRS IEPLKVALQD
sbyvir  SEQGVRVDVL .......... ...VNVSELA EVAAPFVERT IKIVKEVYEK

        401                                                  450
    Ma  ....AKIDKN QVDEIVLVGG STRIPRIQKL ITEYFNGKQP SKSINPDEAV
    Nc  ....AKIDKS QVHEIVLVGG STRIPRIQKL ISDYFNGKEP NKSINPDEAV
    Pg  ....AKIDKA AVHEIVLVGG STRIPRIVKL VSDFFNGKEP NKSINPDEAV
  Ssa1  ....AKLDKS QVDEIVLVGG STRIPKVQKL VTDYFNGKEP NRSINPDEAV
    Ca  ....AKIDKS QVEEIVLVGG STRIPKIQKL VSDFFNGKEL NKSINPDEAV
  Ssa2  ....AKLDKS QVDEIVLVGG STRIPKVQKL VTDYFNGKEP NRSINPDEAV
    Be  ....AKMAKN EVHEIVLVGG STRIPRIQKL VSDFFNGKEP NKSINPDEAV
    Dd  ....SKLDKK SIHEIVLVGG STRIPKVQQL LQEFFNGKEL NKSINPDEAV
    Ss  ....AKLDKA QIHDLVLVGG STRIPKVQKL LQDFFNGRDL NKSINPDEAV
    Ak  ....SKLSKS QVHEVVLVGG STRIPKVQQL LSDFFNGKEP CKSINPDEAV
    Km  ....AKISKS QIDEVVLVGG STRIPKVQKL LSDFFDGKQL EKSINPDEAV
    Sp  ....SNLKKS EIDDIVLVGG STRIPKVQEL LESFF.GKKA SKGINPDEAV
    Hs  ....AEVSKS DIGEVILVGG MTRMPKVQQT VQDLF.GRAP SKAVNPDEAV
    Ec  ....AGLSVS DIDDVILVGG QTRMPMVQKK VAEFF.GKEP RKDVNPDEAV
sbyvir  YCSSMRLEPN VKAKLLMVGG SSYLPGLLSR LSSIPFVDEC LVLPDARAAV
```

```
         451                                                  500
    Ma   A..........  ..........  ..........  ..........  ..........
    Nc   AYGAAVQAAI LSGDTSSKST SEILLLDVAP LSLGIETAGG MMTKLIPRNT
    Pg   AYGAAVQAAI LTGDTSEK.T QDLLLLDVSP LSLGIETAGG VMTPLIKRNT
  Ssa1   AYGAAVQAAI LTGDESSK.T QDLLLLDVAP LSLGIETAGG VMTKLIPRNS
    Ca   AYGAAVQAAI LTGDTSSK.T QDILLLDVAP LSLGIETAGG IMTKLIPRNS
  Ssa2   AYGAAVQAAI LTGDESSK.T QDLLLLDVAP LSLGIETAGG VMTKLIPRNS
    Be   AYGAAVQAAI LAGDQSEK.V QDLLLLDVAP LSLGIETAGG VMTPLIKRNT
    Dd   AYGAAVQAAI LSNEGGAK.V ADLLLLDVAP LSMGLETAGG VMTTLIPRNT
    Ss   AYGAAVQAAI LMGDKSEN.V QDLLLLDVAP LSLGLETAGG VMTALIKRNS
    Ak   AFGATVQAAI LSGNDSSEKL QDLLLLDVTP LSLGLETAGG VMTTLIQRNT
    Km   AYGAAVQGAI LTGQSTSDET KDLLLLDVAP LSLGVGMAGD VFGVVVPRNT
    Sp   AYGAAVQAGV LSGEEGS... DNIVLLDVIP LTLGIETTGG VMTKLIGRNT
    Hs   AIGAAIQGGV LAGD.....V TDVLLLDVTP LSLGIETLGG VFTKLINRNT
    Ec   AIGAAVQGGV LTGD.....V KDVLLLDVTP LSLGIETMGG VMTTLIAKNT
sbyvir   AGGCALYSAC LRNDSP.... ..MLLVDCAA HNLSISSKYC ESIVCVPAGS

         501                                                  550
    Ma   ..........  ..........  ..........  ..........  ..........
    Nc   TIPTKKSEVF STFSDNQPGV L.IQVYEGER QRTKDNN..L LGKFELTGIP
    Pg   TVPTKKSEIF STYSDNQPGV L.IQVYEGER ARTKDNN..L LGKFELAGIP
  Ssa1   TIPTKKSEIF STYADNQPGV L.IQVFEGER AKTKDNN..L LGKFELSGIP
    Ca   TIPTKKSETF STYADNQPGV L.IQVFEGER AKTKDNN..L LGKFELSGIP
  Ssa2   TIPTKKSEVF STYADNQPGV L.IQVFEGER AKTKDNN..L LGKFELSGIP
    Be   TIPAKKSETF STYADNQPGV L.IQVYEGER ARTKDNN..L LGKFELTGIP
    Dd   TIPCKKTQTF STYSDNQPGV L.IQVYEGER AMTKDNN..L LGKFELSGIP
    Ss   TIPTKQTQIF TTYSDNQPGV L.IQVYEGER AMTRDNN..L LGRFELSGIP
    Ak   TVPTKKSQTF STYADNQPGV L.IQVFEGER SMTRDNN..L LGKFSLDGIP
    Km   TVPTIKRRTF TTVADHQTTV T.FPVYQGER VNCKENT..L LGEFDLKGVP
    Sp   PIPTRKSQIF STAVDNQNTV L.IQVYEGER TLTKDNN..L LGKFDLRGIP
    Hs   TIPTKKSQVF STAADGQTQV E.IKVCQGER EMAGDNK..L LGQFTLIGIP
    Ec   TIPTKHSQVF STAEDNQSAV T.IHVLQGER KRAADNK..S LGQFNLDGIN
sbyvir   PIPFTGVRTV NMTGSNASAV YSAALFEGDF VKCRLNKRIF FGDVVLGNVG

         551                                                  600
    Ma   ..........  ..........  ..........  ..........  ..........
    Nc   PAPRGVPQIE VTFDVDANGI MNVSALEKGT .GKTNQITIT NDKGRLSKEE
    Pg   PAPRGVPQIE VTFDIDANGI LNVSASDKTT .GKSNRITIT NDKGRLSKEE
  Ssa1   PAPRGVPQIE VTFDVDSNGI LNVSAVEKGT .GKSNKITIT NDKGRLSKED
    Ca   PAPRGVPQIE VTFDIDANGI LNVSALEKGT .GKTQKITIT NDKGRLSKEE
  Ssa2   PAPRGVPQIE VTFDVDSNGI LNVSAVEKGT .GKSNKITIT NDKGRLSKED
    Be   PAPPRVPQIE VSFDVGADGI LNVSAVDKST .NRSNKITIT NDKGRLSKEE
    Dd   PAPRGVPQVE VTFDVDANGI LNVSAEDKST .GNKQKITIT NDKGRLSKEE
    Ss   PAPRGVPQIE VTFDIDANGI LNVTATDKST .GKANKITIT NDKGRLSKEE
    Ak   PMPRGVPQID VTFDIDANGI LNVSAVEKST .GKENKITIT NDKGRLTKDD
    Km   PMPAGEPVLE AIFEVDANGI LKVTAVEKST .GKSANITIS NAIGRLSSEE
    Sp   PAPRGVPQIE VTFEVDANGV LTVSAVDKSG KGKPEKLVIK NDKGRLSEED
    Hs   PAPRGVPQIE VTFDIDANGI VHVSAKDKGT .RREQQIVIQ SSGG.LSKDD
    Ec   PAPRGMPQIE VTFDIDADGI LHVSAKDKNS .GKEQKITIK ASSG.LNEDE
sbyvir   VTGSATRTVP LTLEINVSSV GTISFSLVGP TGVKKLIGGN AAYDFSSYQL
```

```
       601                                                    650
   Ma  .......... .......... .......... .......... ..........
   Nc  IERMLAEAEK FKEEDEAEAK RVAAKNGLES YAYSLRNTL. SDSKVDEKLD
   Pg  IERMVNEAEK YKAEDEEATA RITSRNALES YAYNLRNSL. TDEKLADKFD
 Ssa1  IEKMVAEAEK FKEEDEKESQ RIASKNQLES IAYSLKNTI. SEAG..DKLE
   Ca  IDKMVSEAEK FKEEDEKEAA RVQAKNQLES YAYSLKNTI. NDGEMKDKIG
 Ssa2  IEKMVAEAEK FKEEDEKESQ RIASKNQLES IAYSLKNTI. SEAG..DKLE
   Be  IERMVAEAEK YKKEDEEAAS RIQAKNGLES YAYNLRNTL. NDDKVAGKMD
   Dd  IEKMVADAEK FKQQDEQQKD RVESKNKLEN YAFTVKNSI. KDEKVAAKIS
   Ss  IERMVQEAEK YKAEDEIQRE RVGAKNALES YAFNMKSVV. EDEGLKGKIS
   Ak  IERMVQEAEK YKSEDEANNV RIEAKNGLEN YAYNLRNTL. NDEKLQGKID
   Km  IEQMVNQAEE FKAADEAFAK KHEARQRLES YISSVQQTV. TDPVLSAKIK
   Sp  IERMVKEAEE FAEEDKILKE RIEARNTLEN YAYSLKGQFD DDEQLGGKVD
   Hs  IENMVKNAEK YAEEDRRKKE RVEAVNMAEG IIHDTETKM. ..EEFKDQLP
   Ec  IQKMVRDAEA NAEADRKFEE LVQTRNQGDH LLHSTRKQV. ..EEAGDKLP
sbyvir GERVVADLHK HNSDKVK... .......... ....LIHALT YQPFQRKKLT

       651                                                    700
   Ma  .......... .......... .......... .......... ..........
   Nc  AADKEKLKSE IDKIVAWLD. ENQQATREEY EERQKELEAI ANPIMMKFYG
   Pg  AADKKKLEDA VNSTISWLD. NSQEASKEEY EEHQKELEAV ANPIMQKLYA
 Ssa1  QADKDTVTKK AEETISWLD. SNTTASKEEF DDKLKELQDI ANPIMSKLY.
   Ca  ADDKEKLTKA IDETISWLD. ASQAASTEEY EDKRKELESV ANPIISGAYG
 ssa2  QADKDAVTKK AEETIAWLD. SNTTATKEEF DDQLKELQEV ANPIMSKLY.
   Be  AADKETLNKA IDETISWLD. GNQEGAKDEY EHKQKELEGV ANPIMTKLYS
   Dd  DSDKSTIESE TESVLKWLE. SNQTAEKDEY EDKMKALEAV VNPIMSKLYQ
   Ss  EADKKKVLDK CQEVISWLD. ANTLAEKDEF EHKRKELEQV CNPIISGLYQ
   Ak  ESDKKVIDDK VTDIINWLD. HNQSAEKEEF EAKQKELEGI ANPIMQKMYA
   Km  RNAKAKVEAA LADAFSTLQ. IEDASADDLR KAEVGLKRAV TKAMSTR...
   Sp  PEDKQAVLDA VEDVAEWLEI HGEDASKEEF EDQRQKLDAV VHPITQKLYS
   Hs  ADECNKLKEE ISKMRELLA. RKDSETGENI RQAASSLQQA SLKLFEMAYK
   Ec  ADDKTAIESA LTALETALK. GEDKAAIEAK MQE...LAQV SQKLMEIAQQ
sbyvir DGDKALFLKR LTADYRREAR KFSSYDDAVL NSSELLLGRI IPKILRGSRV

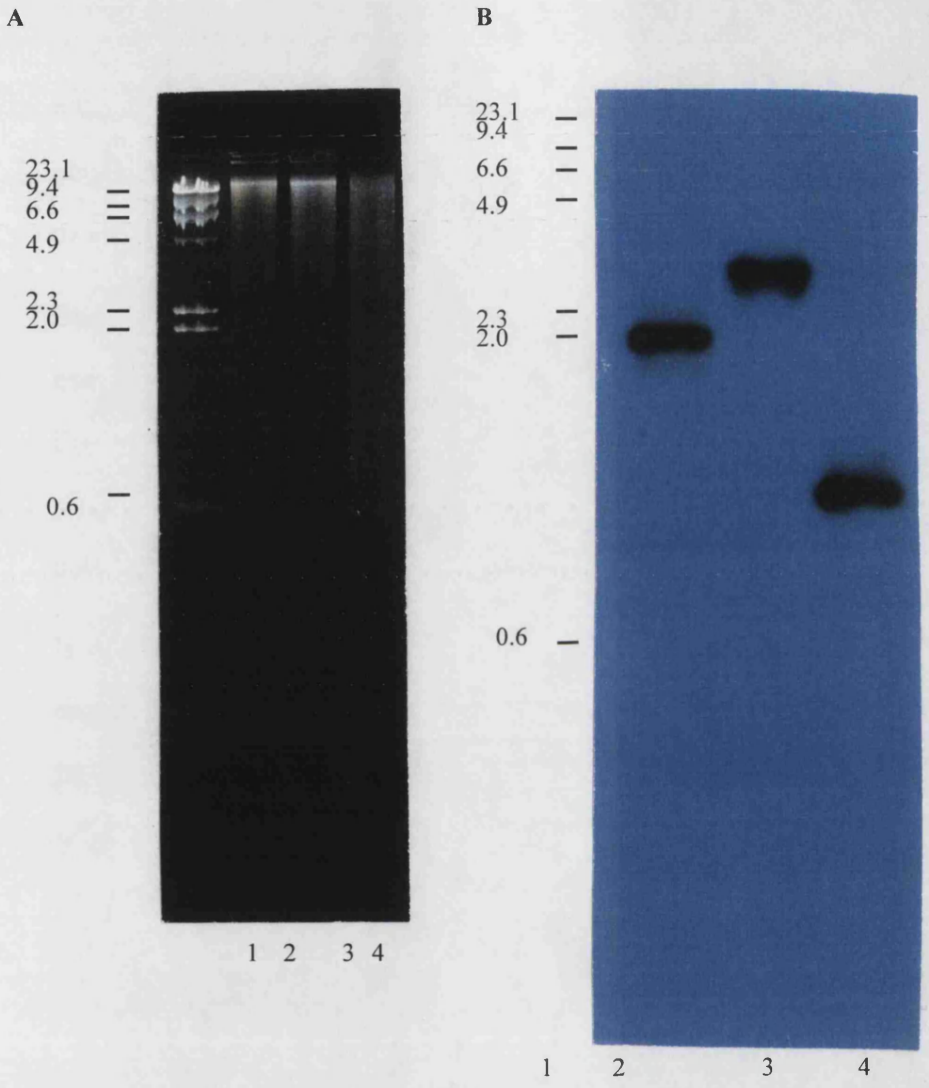
       701                                                746
   Ma  .......... .......... .......... .......... ......
   Nc  .AGGAPGGMP G.AAPGGFPG GAPGS..... ...NDNEGPT VEEVD*
   Pg  GAGGAPGGAP GGF.PGGAPG GFPGGA.... ..PAGEDGPS VEEVD*
 Ssa1  QAGGAPGGAA G.GAPGGFPG GAPPA..... ...PEAEGPT VEEVD.
   Ca  AAGGAPGGAG GFPGAGGFPG GAPGAGGPGG ATGGESSGPT VEEVD.
 Ssa2  QAGGAPEGA. ...APGGFPG GAPPA..... ...PEAEGPT VEEVD.
   Be  AAGGAPGGMP GGFDPSGAP. ....PP.... ..AADTTGPT IEEVD.
   Dd  EGGMPQGGGM PGGMSNDSP. .......... ....KSSNNK VDELD.
   Ss  GAGGPGPGGF GAPDLKGGSG S......... .......GPT IEEVD.
   Ak  AAGGAPGGMP GGMPDMGGAG APPPA..... ...SHAQGPK IEEVD.
   Km  .......... .......... .......... .......... ......
   Sp  EGAGDADEED DDYFDDEADE L......... .......... ......
   Hs  KMASEREGSG SSGTGEQKED QKEEKQ.... .......... ......
   Ec  QHAQQQTAGA DASANNAKDD DVVDAEFEEV KDKK...... ......
sbyvir EKLDV..... .......... .......... .......... ......
```

Alignment of HSP70 deduced amino acid sequences from a range of organisms, ranked according to their percentage identity with the deduced amino acid sequence from the *hsp70* PCR product from Ma - *M. anisopliae* isolate ME1 (letters in bold represent sequence from the primers). Details of the codes used for each organism are given in Table 4-13.

Table 4-13. Details of organisms used in the deduced amino acid alignment against the *hsp70* PCR product from *Metarhizium anisopliae* isolate ME1.

Code	Species	Protein	% identity	Accession number	Mr.
Nc	*Neurospora crassa*	HSP70	82.0%	u10443	-
Pg	*Puccinia graminis*	HSS1	76.8%	u26597	-
Ssa1	*Saccharomyces cerevisiae*	Ssa1	76.7%	p10591	69.5
Ca	*Candida albicans*	HSP70	76.5%	p41797	70.2
Ssa2	*Saccharomyces cerevisiae*	Ssa2	76.4%	p10592	69.3
Be	*Blastocladiella emersonii*	HSP70	75.9%	p48720	70.8
Dd	*Dictyostelium discoideum*	HSC70	74.0%	p36415	70.5
Ss	*Sus scrofa*	HSP70	73.0%	p34930	70.1
Ak	*Achyla klebsiana*	HSP70	72.4%	p41753	71.3
Km	*Kluyveromyces maxianus*	Ssb1	66.4%	p41770	66.0
Sp	*Schizosaccharomyces pombe*	BiP	54.8%	p36604	73.1
Hs	*Homo sapiens*	Grp75	54.4%	p38646	73.8
Ec	*Escherishia coli*	DNAK	51.1%	p04475	68.9
sbyv	sugar beet yellow virus	HSP70 homologue	23.9%	p37092	65.2

Figure 4-18. Screening of a Southern blot of restricted genomic *Metarhizium anisopliae* isolate ME1 DNA with ^{32}P the labeled *hsp70* PCR fragment.



A, agarose gel with restricted genomic *M. anisopliae* isolate ME1 DNA; **B**, probed Southern blot of A. Lane **1**, HindIII restricted λ marker with sizes marked in kb; lane **2**, restricted with Ast 718; lane **3**, restricted with BamHI; lane **4**, restricted with Hind III.

4.3.3.5 Screening the *Metarhizium anisopliae* λ library for *hsp70* genes

Having established that the PCR product cloned was an *hsp70* gene from *M. anisopliae* this fragment was then used to screen the λ library from this organism. This was done so that any further work carried out on this gene could be done with the genomic DNA rather than the PCR product which may have mismatched base pairs. Isolation of the genomic DNA also allows sequencing of the upstream region and hence identification of regions involved in control of transcription such as HSEs.

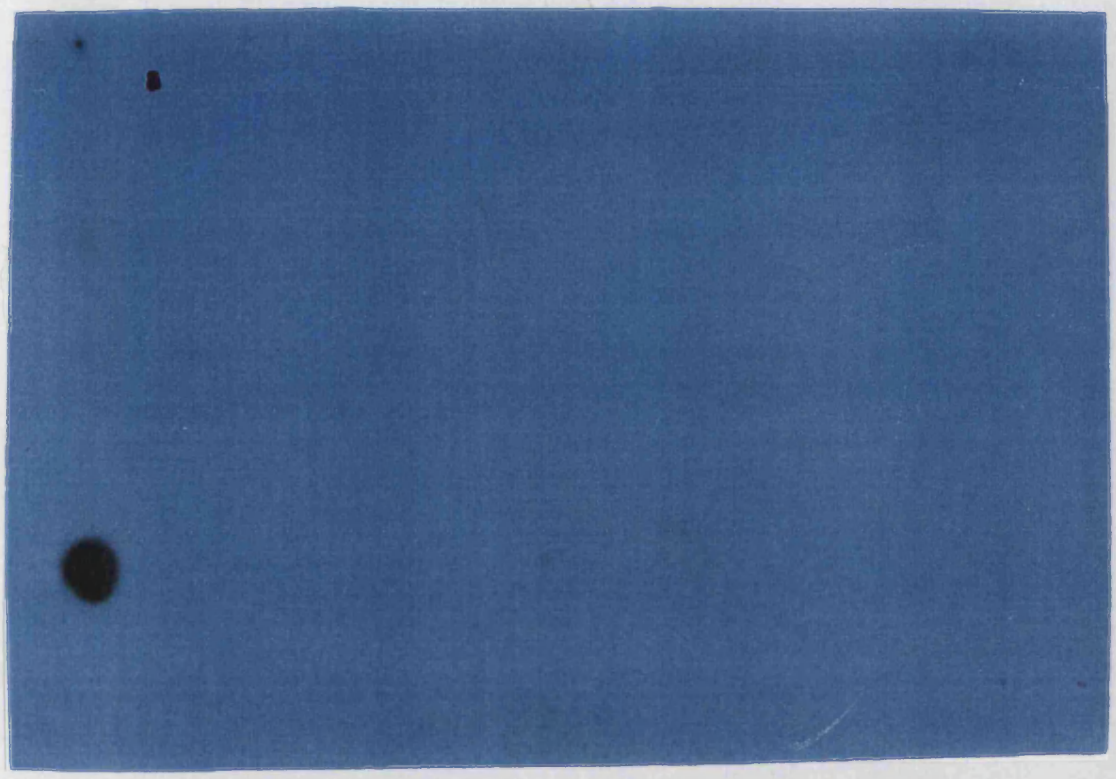
Based on the dilution series, the *M. anisopliae* isolate ME1 λ library the titre was estimated at 421 pfu μl^{-1}. An estimated 15000 plaques were screened from four $20cm^2$ Petri dish plates using the cloned fragment. These screens revealed only one positive plaque giving further evidence that in *M. anisopliae* the *hsp70* is a very low copy number gene (Figure 4-19).

Isolation and restriction of the λ clone from this plaque with a number of restriction enzymes gave a number of different bands (Figure 4-20) which were then blotted and probed again with the *hsp70* PCR product. Single bands of lit up with in all lanes (Figure 4-21).

The *hsp70* gene was not cloned from the *M. anisopliae* λ library during this project as there was not sufficient time.

Figure 4-19. Screening of genomic *Metarhizium anisopliae* isolate ME1 λ library with ^{32}P labeled *hsp70* PCR fragment.

Primary screen



Secondary screen

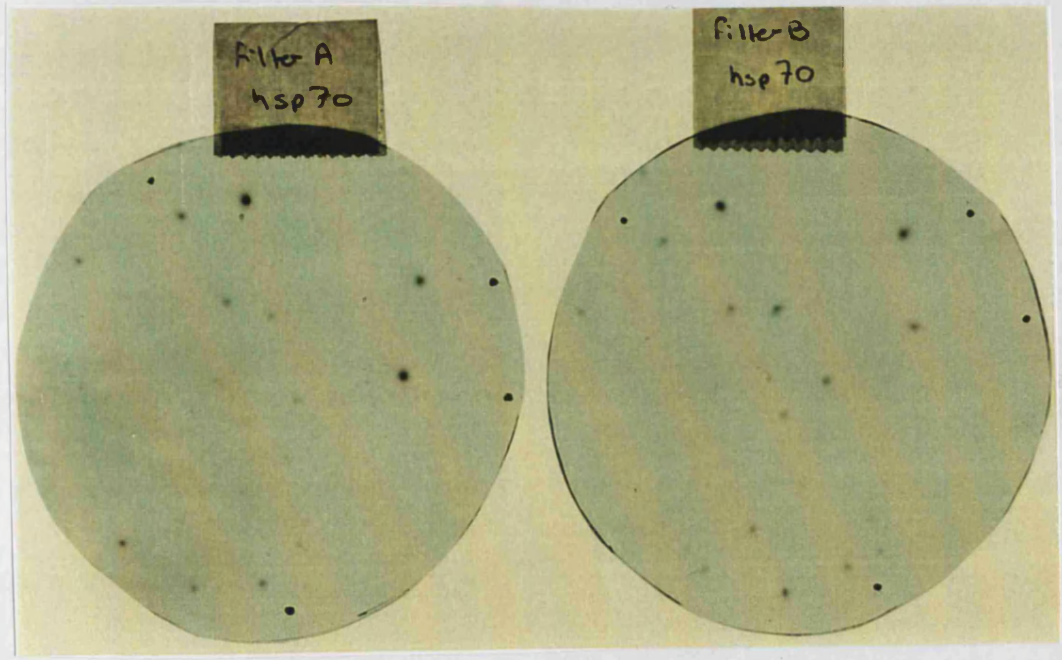
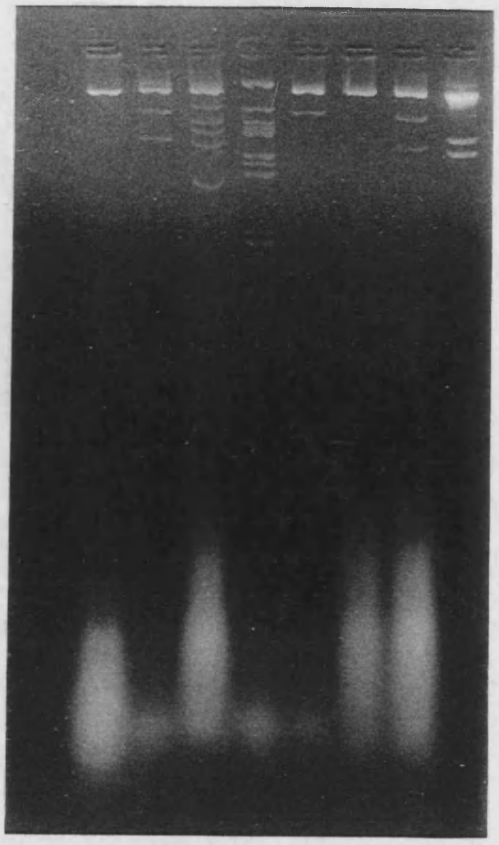
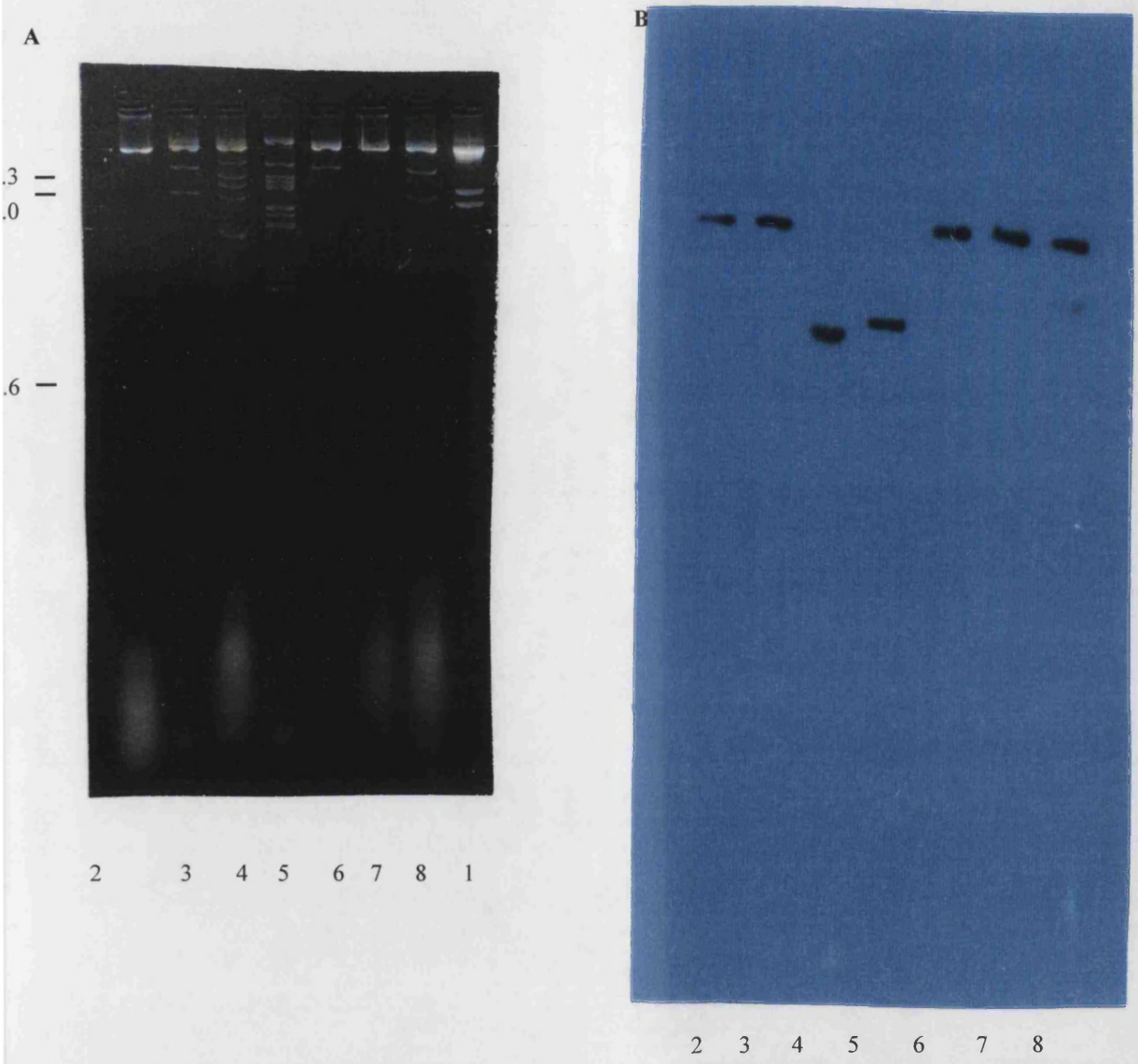


Figure 4-20. Restriction digests of λ clone isolated with *hsp70* PCR probe.



Lane **1**, HindIII cut λ marker; lane **2**, uncut *hsp70* λ clone; lanes 3 - 8 *hsp70* λ clone restricted with: lane **3**, XhoI; lane **4**, SalI; lane **5**, PstI; lane **6**, EcoRI; lane **7**, BamHI; lane **8**, EcoRI and BamHI.

Figure 4-21. Southern blot of restricted λ clone probed with radio-labeled *hsp70* PCR product from *Metarhizium anisopliae* isolate ME1.



A: agarose gel with restricted *hsp70* λ clone. **B**: probed Southern blot of A. Lane **1**, HindIII cut λ marker; lane **2**, uncut *hsp70* λ clone; lanes 3 - 8 *hsp70* λ clone restricted with: lane **3**, XhoI; lane **4**, SalI; lane **5**, PstI; lane **6**, EcoRI; lane **7**, BamHI; lane **8**, EcoRI and BamHI.

4.3.3.6 Amplification of *hsp70* from *Metarhizium flavoviride* isolate IMI 330189 using the PCR.

The *hsp70* primers were also used in PCR with genomic *M. flavoviride* isolate IMI 330189 DNA. Here nested PCR lead to the production of three different products of approximately 1.3, 1.2 and 1.1 kb (Figure 4-22).

When a Southern blot of the nested PCR products produced by *M. flavoviride* was probed with the cloned *hsp70* gene from *M. anisopliae* all three bands lit up .

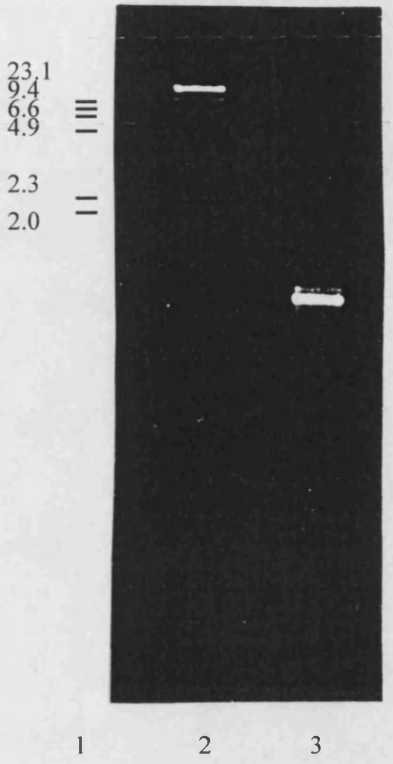
4.3.3.7 Copy number of the *hsp70* gene within *Metarhizium flavoviride* isolate IMI 330189

Probing of a Southern blot of restricted genomic *M. flavoviride* DNA with the *M. anisopliae hsp70* clone under the same conditions used previously to probe for copies the gene in *M. anisopliae,* 1 band of the *M. flavoviride* genomic DNA lit up in each lane (data not shown).

4.3.3.8 *hsp90*

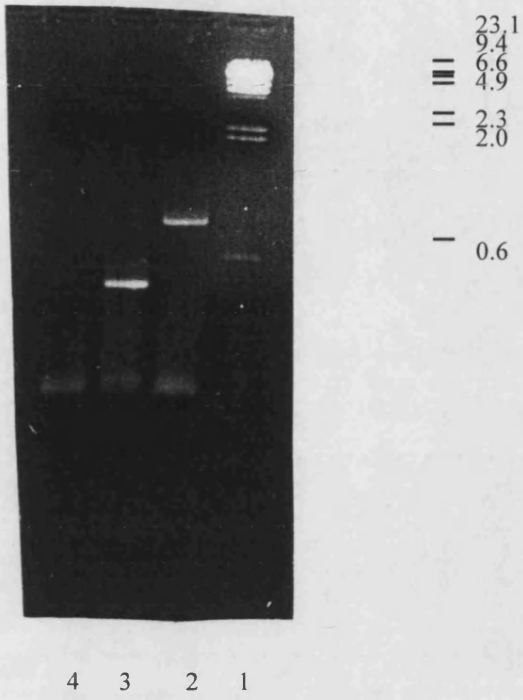
PCR of genomic *M. anisopliae* DNA using the *hsp90* primers lead to the production of single products with each combination of primers used (Figure 4-23). These products were approximately the size expected for amplification of the *hsp90* gene. A southern blot of restricted genomic *M. anisopliae* DNA with the larger putative *hsp90* PCR product revealed the presence of one reactive band in each lane, indicating that this is probably a single copy gene also.

Figure 4-22. Products amplified by the polymerase chain reaction from genomic *Metarhizium flavoviride* isolate IMI 330189 DNA using the *hsp70* oligonucleotide primers.



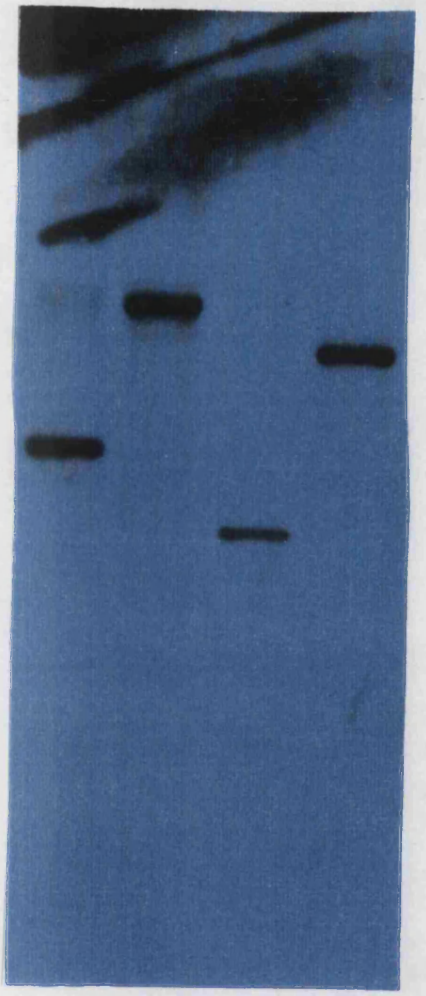
PCR conditions are listed in methods. Lane **1**, HindIII restricted λ marker; lane **2**, PCR products produced between oligonucleotides *hsp70-1* and *hsp70-3*; lane **3**, PCR products produced between oligonucleotides *hsp70-1* and *hsp70-4*; lane **4**, PCR products produced between oligonucleotides *hsp70-2* and *hsp70-3*; lane **5**, PCR products produced between oligonucleotides *hsp70-2* and *hsp70-4*, lane **6**, nested PCR product; lane **7**, control reaction with no DNA; lane **8**, cleaned nested PCR product.

Figure 4-23. Products amplified by PCR using the *hsp90* oligonucleotide primers to amplify genomic *Metarhizium anisopliae* ME1 DNA.



PCR conditions are listed in Methods. Lane **1**; HindIII restricted λ marker, lane **2**; PCR products produced between oligonucleotides *hsp90-1* and *hsp90-3*; lane **3** PCR products produced between oligonucleotides *hsp90-2* and *hsp90-3*; lane **4** control reaction with no DNA.

Figure 4-24. Southern blot of genomic *Metarhizium anisopliae* isolate ME1 with ^{32}P labeled putative *hsp90* PCR product.



Genomic *M. anisopliae* was digested with lane **1**, *Bam*HI; lane **2**, *Eco*RI; lane **3**, *Pst*I and lane **4**, *Sal*I

4.3.3.9 Transcription of *hsp70* in *Metarhizium flavoviride* isolate IMI 330189

Total RNA was extracted from control, heat shocked, starved and cuticle grown cultures with the RNeasy QIAGEN kit. The quantity and purity of the RNA was checked using spectrophotometry and by running on a formaldehyde gel. The results from the RNA extraction are shown in Table 4-14 and Figure 4-25.

When 10 μg of each sample were run on a gel with an RNA standard they gave two visible bands of about 2.4 kb and 4 kb (Figure 4-25).

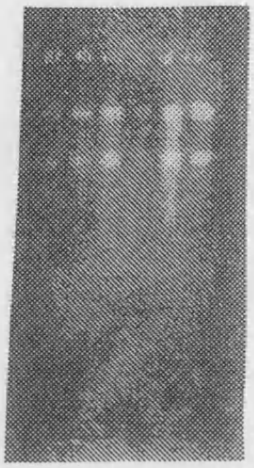
These RNA samples were used in perform RT-PCR as described in the methods. These showed 3 different PCR products corresponding to three different transcribed products (Figure 4-26). Virtually no transcribed product was detected in the control culture (a small amount of the intermediate sized product was detected although this cannot be seen on the photograph). All three products appeared to be induced by one hours exposure to 45°C, particularly the middle band. Starvation appeared to lead to almost complete repression of all product production except for the largest. Cuticle culture induced the high and low molecular weight products.

Table 4-14. Concentration and purity of extracted RNA

Sample	Concentration ng/µl	A_{260}/A_{280} [A]
Non-heat shocked	192	1.40
Heat shocked	184	1.35
Non-starved	192	1.41
Starved	656	1.58
Glucose	232	1.61
Cuticle	192	1.71

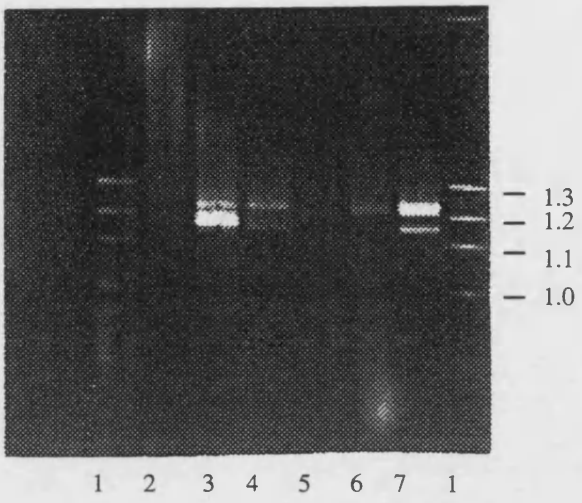
3 day *M. flavoviride* culture grown on Czapek Dox as described in the methods, RNA extracted as described in methods after: **non-heat shocked**; 1 hour in 27 °C shaking water bath; **heat shocked**; 1 hour in 45 °C shaking water bath; **non-starved**; transfer to fresh Czapek Dox for 24 hours; **starved**; transfer to minimal media for 24 hours; **glucose**; transfer to minimal media supplemented with 10% glucose and nitrogen source for 48 hours; **cuticle**; transfer to minimal media supplemented with 10% locust cuticle (in sterile muslin bag to aid separation) for 48 hours. [A] Pure RNA should have a value of between 1.7 - 2.0.

Figure 4-25. Total RNA extracts from *Metarhizium flavoviride* isolate IMI 330189 cultures



Lane **1**; non-heat shocked; 1 hour in 27 °C shaking water bath; lane **2**, heat shocked; 1 hour in 45 °C shaking water bath ; lane **3**, non-starved; transfer to fresh Czapek Dox for 24 hours; lane **4**, starved; transfer to minimal media for 24 hours; lane **5**; glucose; transfer to minimal media supplemented with 10% glucose and nitrogen source for 48 hours; lane **6**, cuticle; transfer to minimal media supplemented with 10% locust cuticle (in sterile muslin bag to aid separation) for 48 hours.

Figure 4-26. RT-PCR from total RNA extracted from *Metarhizium flavoviride* IMI 330189.



Lane **1**; molecular wieght marker; lane **2**; non-heat shocked; 1 hour in 27 °C shaking water bath; lane **3**, heat shocked; 1 hour in 45 °C shaking water bath ; lane **4**, non-starved; transfer to fresh Czapek Dox for 24 hours; lane **5**, starved; transfer to minimal media for 24 hours; lane **6**; glucose; transfer to minimal media supplemented with 10% glucose and nitrogen source for 48 hours; lane **7**, cuticle; transfer to minimal media supplemented with 10% locust cuticle (in sterile muslin bag to aid separation) for 48 hours.

4.4 Discussion

4.4.1 Thermotolerance

Initial experiments investigating thermotolerance in *M. flavoviride* IMI 330189 conidia revealed that pre-exposure to an intermediate heat shock of 45°C had subsequent protective effects when the conidia were exposed to a severe heat shock of 50°C and lead to a 4 fold increase in the survival rate. Addition of cycloheximide to the conidia removed the ability of the intermediate heat shock to induce thermotolerance, thus indicating that in *M. flavoviride* protein synthesis is essential for the acquisition of thermotolerance. These results are very similar to those described previously for *Neurospora crassa* (Plesofsky-Vig and Brambl, 1985). One important difference between the response of *M. flavoviride* and *Neurospora crassa* to heat shock was that the protective effect of the intermediate heat shock did not appear to decrease as quickly in *M. flavoviride*. Even if the conidia were left at 27°C for up to 3 hours after the intermediate heat shock, they still showed the same level of thermotolerance induction as those spores transferred directly to from the intermediate to the severe heat shock. In *Neurospora crassa* a drop of over 50% in the number of colony forming units is seen over the same period. It is unlikely that the *M. flavoviride* conidia would continue to show this high level of thermotolerance for an extended period and samples taken at later periods would probably reveal a reduction in thermotolerance. However, this apparent long period of induced temperature tolerance may provide very useful as a method of protecting spores from the extreme temperature changes they are likely to face in the field.

Exposure of conidia to 50°C lead to a delayed germination speed, even for those conidia to which a mild heat shock had been used to induce thermotolerance. The reasons for this are not yet known, but it may be that it takes the cell longer to recover from the damage caused at this extreme temperature. It is known that the higher the temperature of the heat shock the longer it takes for normal protein synthesis to resume. It is also likely that the higher the temperature of heat shock, the less the HSPs will be able to

protect proteins against irreversible denaturation and thus the more work the cell will have to do once the source of heat shock is removed in order to destroy aberrant proteins and synthesis new ones. This could mean a delay while the cell synthesis's new proteins and could account for the slower germination rate of conidia exposed to 50°C.

Thermotolerance has been demonstrated across a wide range of organisms, not just fungi but whole mice, *Drosophila melanogaster*, soybean seedlings, slime molds and bacterial cells (Morimoto *et al.*, 1990). However, the importance of protein synthesis and in particular HSPs in the process appears to depend on the organism in question. In *Neurospora crassa* (Plesofsky-Vig and Brambl, 1985) and *Escherichia coli* (Silva *et al.*, 1987) experiments have shown that protein synthesis is essential for thermotolerance as it is for *M. flavoviride*. The strongest evidence for the importance of HSPs in thermotolerance comes from experiments done on *Escherichia coli* strains which have mutations in the heat shock induction gene, these organisms do not induce HSPs in response to high temperatures and show no thermotolerance (Silva *et al.*, 1987). Another piece of indirect evidence for the importance of HSPs in thermotolerance comes from an investigation on a number of *Hydra spp.* It was found that *Hydra oligactis* does not produces any polypeptides in response to heat shock and is unable to gain thermotolerance. This investigation also revealed that the heat shock response is not entirely universal as is frequently reported (Bosch *et al.*, 1988).

However, there is a large body of evidence which points to the fact that although HSPs are probably involved in thermotolerance they are not essential in all organisms. In *Schizosaccharomyces pombe* (De Virgilio *et al.*, 1990), *Saccharomyces cerevisiae* (De Virgilio *et al.*, 1991), and *Neurospora crassa* (Neves *et al.*, 1991) an induction of some degree of thermotolerance can be demonstrated even in the presence of a protein synthesis inhibitor like cycloheximide. This ability to induce thermotolerance in these cases is often attributed to the accumulation of trehalose. *M. flavoviride* showed a small

but significant degree of induced thermotolerance in the presence of cycloheximide which could be due to trehalose or glycerol accumulation, although previous work has shown *M. anisopliae* conidia to contain comparatively little trehalose and low molecular weight polyols (Hallsworth and Magan, 1996).

Storage of *M. flavoviride* conidial plates at 4°C severely reduced the heat shock response and lead to very little survival even at 45°C. However, oil formulations of dried *M. flavoviride* conidia do not show the same response, work has shown that over 80% germination can be seen in conidia which have been exposed to 60°C for 90 minutes (McClatchie *et al.*, 1994). *Bacillus subtilis* spores also show greater heat resistance when suspended in oils. It is thought that the oil protects cells against extremes of temperature through its low water availability and the free fatty acid content (Ababouch *et al.*, 1995). However if the conidia of *M. flavoviride* are not first dried their ability to withstand storage at low temperatures becomes severely impaired (Moore *et al.*, 1995). The same pattern of temperature resilience is seen in *Bacillus cereus* which have been stored on silica gel (Dufrenne *et al.*, 1994). It appears that as long as conidia are stored dry in oil they are in a resting stage and are very resilient to temperature changes. However when the conidia are suspended in water or applied to the cuticle of an insect they begin the processes of germination and become much more susceptible to temperature changes. This unfortunately means infection levels at higher temperatures cannot be increased simply by giving the oil suspensions a heat pre-treatment prior to application as has been shown in the results in Chapter 3. Other methods of increasing the ability of the fungus to infect at higher temperatures will have to be found. This could involve manipulation of growth conditions prior to harvesting the conidia to increase not only quantities of heat shock proteins but also other chemicals that protect the fungus from stress (such as glycerol, mannitol and trehalose). Some methods of increasing the concentration of glycerol in *M. anisopliae* to improve germination at low water activities have already been described (Hallsworth and Magan, 1995). Alternatively, if heat shock was to be used to induce thermotolerance and

improve infectivity at higher temperatures it would have to be induced at some time before the before the formulation is made. When this might be can only be discovered by further experimentation. Experiments have shown that HSPs can be induced to accumulate prior to the formation bacterial spores by heat shock to cells at the end of the exponential growth phase, however timing of the heat shock is critical (Sedlak *et al.*, 1993).

IIBC are currently starting to investigate the use of oil/water emulsions (Bateman, pers. comm.) which may allow the fungi to be successfully heat shocked and induce HSPs or other protective proteins/chemicals.

Temperature is often a problem with biological control agents, such as the insect-parasitic nematode, *Heterorhabditis bacteriophora*. Studies have been done with this nematode that show induced thermotolerance will improve survival and infectivity at temperatures which normally inhibited their activity. This experiment found a correlation between the level of HSP70 and thermotolerance. Another interesting point in this study was that it was the first report of heat shock protein and thermotolerance induction in a non-feeding, developmentally arrested organism (Selvan, *et al.*, 1996). Increased infectivity following heat shock has also been obtained with *Leishmania braziliensis* (Smejkal *et al.*, 1988)

4.4.2 Protein production

Having established that thermotolerance could be induced in *M. flavoviride* and that it was due to the manufacture of proteins during heat shock, the production of proteins during the heat shock response was then investigated. This work was carried out on mycelial cultures in order to make comparisons with published data.

There were no detectable differences in the banding patterns of Coomassie blue stained gels of normal and heat shocked intracellular mycelial protein extracts. Other papers have also found that the identification of heat induced polypeptides is not always

possible by this method (Kapoor and Lewis, 1987). Often 2-dimensional electrophoresis is used to help identify proteins induced by heat shock, this method was attempted during this study but was not successful. However, this method has been used successfully to look at the heat shock response of another entomopathogen, *Beauveria brogiariti*. This investigation described three sets of proteins, those induced, repressed and enhanced (Xavier and Khachatourians, 1996).

Due to the problems of identifying polypeptides induced during heat shock, two other methods were used to study the heat shock response. These involved radio-labeling of proteins produced during heat shock and probing Western blots with an antibody to detect the presence of HSP70 related proteins.

In *M. flavoviride* isolate IMI 330189 heat shock only led to an increase in the detection of HSP70-like protein levels at 32°C, all higher temperatures led to a decrease in the amount detected. This appears to be a very unusual response and there is very little information in the literature on heat shock leading to a decrease in the amount of HSP70 present. Only one report has been found of a reduction of the levels of HSP70 on heat shock and this is in the parasite, *Trypanosoma cruzi*. It was found that if stationary phase organisms were heat shocked for two hours at 42°C there was a reduction in the quantity of HSP70 present compared with those kept at the normal growth temperature of 28°C (Requena *et al.*, 1992). Other papers have reported that individual members of the HSP70 family may be degraded by heat treatment, such as the Ssb1 protein in *Saccharomyces cerevisiae*. This protein has been found to be induced at lower temperatures (Iwahashi *et al.*, 1995). The level of induction of the HSP70-like polypeptide also seemed quite low. However, similar patterns of induction have been found for one of the *Caenorhabditis elegans hsp70*'s, where the gene is expressed abundantly under normal conditions but its expression is increased only 2-6 fold on heat shock (Snutch *et al.*, 1988). Perhaps the abundance of constitutively produced HSP70-like proteins means that *M. flavoviride* already has a naturally high level of innate

thermotolerance. It may also be that in *M. flavoviride* HSP70 is only important at mild heat shock temperatures and that at temperatures of 36°C and above other HSPs become more important. In yeast HSP104 is reported as being responsible for thermotolerance at extreme high temperatures and the possible role of this protein in *M. flavoviride* is discussed later.

Western blots of *M. flavoviride* isolate IMI 330189 also showed the production of a 40kDa immuno-reactive polypeptide during exposure to the lower heat shock temperatures of 32, 36 and 40°C but not at 45°C. This protein was not detected in mycelia which had not been heat shocked. A similar 40kDa HSP70 degradation product has been discovered in *Neurospora crassa*, but interestingly in this organism highest levels were found to be present in non-heat shocked mycelia, with a sharp decrease in the levels of the breakdown product detected after heat shock. It was proposed that in this organism the decrease in the 40kDa HSP70 fragment was due to a decrease in ATP-independent proteolysis which coincided with heat shock (Mohsenzadeh *et al.*, 1994). It was proposed that this allowed the HSPs to be more thermostable. During this decrease of ATP-independent proteinases in *Neurospora crassa* it has been found that ATP-dependent proteinases are more important and may even be enhanced by heat shock. In *M. flavoviride* isolate IMI 330189 it may be that ATP-dependent proteinases are responsible for breaking down HSP70 and are induced during heat shock. However, if this was the case it would mean that there would be a higher turn-over of HSP70 during heat shock. Alternatively, the detected polypeptide may be another heat shock inducible polypeptide which is related to HSP70.

In a Western blot of cultures with varying pathogenicity to *S. gregaria* large differences in the pattern of HSP70-like protein production were seen only between the *M. flavoviride* and *M. anisopliae* isolates, but not between the two *M. anisopliae* isolates. Previous experimenters have reported that HSP70 levels induced by heat shock can be correlated to virulence in *Toxoplasma gondi* (Lyons and Johnson, 1995). With the two

M. anisopliae isolates there were no differences detected in levels HSP70 despite differences in virulence against *S. gregaria*. However, this is perhaps not surprising as neither of these two isolates were isolated from *S. gregaria* and therefore their virulence against this organism is somewhat of an artificial classification. It would be interesting to repeat this experiment using only isolates of *Metarhizium spp.* which had been isolated from *S. gregaria* in order to investigate the relationship between virulence and HSP70 production.

All the *Metarhizium* species investigated by ^{35}S labeling produced a large quantity of an 106kDa polypeptide in response to heat shock which could be a protein from the HSP100 family. In yeast, HSP104 is frequently associated with thermotolerance acquisition. It is known from studies on mutants lacking the HSP104 gene that its presence helps the organism survive extremes of temperature stress. Often this protein is undetectable during normal growth conditions but becomes the major protein after a shift to growth at high temperatures (Parsell *et al.*, 1993). The roles of HSP100 are extremely highly conserved and plant HSP100 complements mutants of yeast which are deficient in HSP100 (Lee *et al.*, 1994; Schirmer *et al.*, 1994). In addition HSP104 is found in very high levels in spores of *Saccharomyces cerevisiae* and is crucial for their naturally high thermotolerance and ability to retain long-term viability at low temperatures. From preliminary data gathered during this study it would appear that the 106kDa polypeptide found in heat shocked *M. flavoviride* may be an HSP100 and may therefore have a similar role in protecting spores from temperature increases and enabling them to germinate.

HSP104 from *Saccharomyces cerevisiae* rescues heat damaged proteins by re-solubilising those which have formed aggregates. In this way it is thought to complement HSP70 which prevents aggregates of proteins forming (Parsell *et al.*, 1994). Not all organisms produce an HSP100. It appears that in such organisms, like *Drosophila melanogaster*, HSP70 production correlates with acquired thermotolerance

(Parsell *et al.*, 1993). Other HSPs have also been implicated in thermotolerance, but are usually induced as well as HSP100 or HSP70 (Plesofsky-Vig and Brambl, 1995; Kaneko *et al.*, 1995). However, there are a few organisms in which HSP60 is the only protein induced in response to heat shock (Bosch *et al.*, 1988, Yokota *et al.*, 1994).

The sizes and patterns of polypeptide induction by heat shock in *Metarhizium spp.* are very similar to those produced by a number of other organisms. At 32°C there is a major induction of four polypeptides possibly corresponding to 100, 80, 70 and 60kDa HSPs with little or no decrease in overall polypeptide synthesis. At extreme heat shocks of 45°C, the two *M. anisopliae* isolates do not synthesize any proteins, but *M. flavoviride* isolate IMI 330189 produces a single polypeptide of 106kDa. Similar patterns of polypeptide induction have been found in *Leptosphaeria maculans* (Chakraborty *et al.*, 1992), *Neurospora crassa* (Kapoor and Chow, 1984) and *Fonsecaea pedrosoi* (Laurent-Winter and Ibrahim-Granet, 1992).

It has been found with *Drosphila* that the optimum temperature for thermotolerance induction is one which leads to the induction of the HSPs while leaving the overall rate of RNA and protein synthesis only slightly affected. This thermotolerance has been shown to be closely correlated with HSP production, especially with the production of HSP70. In the mycelium of *Metarhizium spp.* it would appear that based on this theory that 32°C would be an ideal temperature for inducing thermotolerance, as there is very little change in protein production but HSP70 induction is at a maximum.

The results from the ^{35}S methionine labeling and Western blots of the proteins produced by different isolates of *Metarhzium spp.* have shown that there are large differences in the induction of proteins between *M. anisopliae* and *M. flavoviride*. It is particularly interesting to note that neither of the *M. anisopliae* isolates produce any proteins at 45 ° C. Such variation between species has been shown in other organisms, an extreme case is demonstrated in two species of *Hydra*. A species from an extremely thermostable environment has no detectable heat shock inducible HSPs whereas the species present in

a broad range of habitats shows strong induction of HSP60 during heat shock (Coleman, *et al.*, 1995). Differences in the environments from which each isolate of *Metarhizium* was found or evolved from may account for the differences in heat-shock response. That is, the two *M. anisopliae* isolates may not be able to produce heat shock proteins at 45 °C as within their environment they are rarely required to cope with this temperature. *M. flavoviride* on the other hand comes from a hotter environment and would therefore be expected to have a higher thermal niche and consequently is more likely to be adapted to survival at 45 °C. However the differences could also be due to host preference. *M. flavoviride* isolate IMI 330189 is pathogenic to grasshoppers and locust and previous work has shown that these insect show a high degree of behavioural fever during infection (M. Thomas, pers. comm.), any entomopathogenic fungus which is going to overcome these extreme temperatures is going to have to show a high degree of thermotolerance. The hosts that the two *M. anisopliae* isolates were isolated from are both likely to have much lower body temperatures. *M. anisopliae* isolate ME1 infects beetles and was isolated from a much cooler and more humid climate than the other two isolates. *M. anisopliae* isolate I91 633 although from a hotter climate was isolated from a cricket which tends to spend most of the day under shelter or in burrows and emerges only at night. This would mean that the two *M. anisopliae* isolates both have less requirement to survive extremes of heat than the *M. flavoviride* isolate and therefore possibly do not require the production HSPs at temperatures as extreme as 45°C. These differences in the abilities of *M. anisopliae* isolate ME1 and *M. flavoviride* isolate IMI 330189 to respond to extremes of temperature also correlates to their growth rate *in vitro* at high and low temperatures as discussed in Chapter 2. In short each isolate of *Metarhizium spp.* must balance the benefits and costs of producing HSPs and produce a response which is going to be most effective for the type of stress that it might encounter. Studies have shown that the repeated induction of thermotolerance in *Drosophila melanogaster* leads to an increased survival of temperature extremes but a

significant reduction in the number of off-spring that individual will have (Krebs and Loeschcke, 1994).

Previous experiments have implicated the importance of heat shock protein synthesis in the virulence of fungal pathogens. Comparisons between a virulent and weakly virulent strain of the plant fungal pathogen, *Leptosphaeria maculans*, showed that the virulent strain had a very pronounced heat shock response, producing many new polypeptides and showing a marked decrease in the production of other polypeptides during heat shock. The weakly virulent strain did not show such a pronounced response, not producing as many new polypeptides and showing sustained synthesis of normal cellular polypeptides even at 45°C (Chakraborty *et al.*, 1992). However, other experiments with *Leptosphaeria maculans* showed that both virulent and avirulent strains have very similar patterns of induction of heat shock proteins throughout development (Patterson and Kapoor, 1995). Studies investigating the heat shock response of virulent and avirulent *Porphyromonas gingivalis*, however, found no differences in the responses of different strains (Vayssier *et al.*, 1994). Sahu *et al.* (1994) also found no difference in the pattern of induction of the HSPs in *Vibrio cholerae* strains with varying levels of toxinogenicity.

Comparisons of the heat shock responses of *Metarhizium spp.* isolates with varying virulence towards *S. gregaria* revealed that all three isolates showed very similar patterns of polypeptide production during heat shock. This was particularly true of the two *M. anisopliae* isolates, ME1 and I91 633, even though they had widely varying virulence against *S. gregaria*. Both showed very similar patterns of heat shock inducible polypeptides at 32°C and failed to produce any detectable polypeptides at 45° C.

Very little differences were found in the proteins present in control and infected haemolymph from male locusts. However, previous work has detected a number of proteins which do significantly alter during the course of infection (Gillespie, 1995).

Here proteins could be classified into three groups, those which stayed the same over the course of infection, those which declined during infection and those which appeared during infection. The reasons for the differences in detection of protein changes between these two studies may be due to the differences in analysis methods or due to differences in the size range of proteins studied. Gillespie studied proteins in the size range of 11 - 70kDa, whereas the protein size range in this study was from 13 - 173kDa. This larger protein size range included a few very abundant proteins (e.g. the 87 kDa protein) and these may have lead to a drop in sensitivity of the detection of differences in less abundant proteins. In addition the dose and time scale of the experiments was different. The Coomassie blue stained gels in this report used a higher inoculum level and as a result were only carried out over three days rather than the four days used in Gillespie's experiments. The ^{35}S methionine experiments, however, were carried out under the same conditions as described by Gillespie.

Experiments on human fungal pathogens have lead to the suggestion that the pathogens HSPs may be a very important factor in virulence. They are often found to be the dominant antigens and have been implicated as potentially causing damage when released into the host. The HSP70-like polypeptides detected in the haemolymph of *S. gregaria* on days two and three of infection are unlikely to be of host origin and have probably come from *M. flavoviride*. Evidence for this is provided by the fact that the HSP70-like protein detected in the haemolymph of infected insects is approximately the same size as the major HSP70 produced by *M. flavoviride*. However, it was difficult to estimate the exact size due to the interference of a large protein just above the HSP70 homologue which caused some distortion. No published data was found on the size of HSP70's in *S. gregaria*. However this information could be obtained by preparing Western blots of protein extracts from heat shocked locusts and probing with the α-HSP70 antibody. HSPs are normally not detected outside of a cell, unless cell lysis occurs and the contents are released. It is possible that the appearance of HSP70 on days two and three of infection is due to the lysis of protoplasts in the locust's

haemolymph due to the action of the insect's defense reaction. Previous work certainly suggests that *M. flavoviride* can be detected in the haemolymph as little as 48 hours after infection (Gillespie, 1995) and by this time there is sufficient fungus in the insect that if haemolymph is transferred from an infected to a non-infected insect, mycosis will follow in the latter (McKemey, 1995). Cobb (1997) has also shown that protoplasts can be detected in *M. anisopliae* infected *Manduca sexta* within 48 hours. On day four it may be that no HSP70-like proteins are detected because by that time the fungus has completely overcome the host's defense mechanisms and therefore the cells are no longer being lysed. Alternatively, it may be that by this time the fungus has largely switched to a walled form and is much less susceptible to lysis. Further evidence for the likelihood of the HSP70-like proteins being of fungal origin comes from the report that infection of human and murine cells with *Leishmania major* induces only the pathogens HSPs and not those of the host (Kantegwa *et al.*, 1995). However, infection has induced the production of host HSPs in other cases. The level of HSP70 detected in the haemolymph was extremely low and other researchers have reported that they have been unable to detect HSP70 produced by pathogens *in vivo* (Lyons and Johnson, 1995). Also HSP70's may not play an important role in infection in all cases. In the plant fungal pathogen, *Leptosphaeria maculans* mRNA from the fungal *hsp70* can be detected in plants infected with either virulent and non-virulent strains (Patterson and Kapoor, 1995). In human fungal pathogens it is often HSP90 which is thought to be the major HSP responsible for damage in the host and further experiments looking at the role of *M. flavoviride*'s HSPs in the haemolymph should perhaps concentrate on this protein.

It is hypothesised by a number of workers that in some pathogenic organisms (e.g. *Toxoplasma gondii*) the induced expression of HSP70 in virulent strains by stresses associated with infection may provide protection for these strains against cell damage associated with invasion of the host. It is proposed that avirulent strains which do not produce this response are then unable to protect themselves against the hosts defenses.

These differences in HSP70 production are sometimes only seen *in vivo* (Lyons and Johnson, 1995).

Expression of fungal pathogens heat shock proteins has also been detected by probing mRNA extracted from wheat plants infected with *Leptosphaeria maculans* for HSP70 and HSP80. Here the authors detected the presence of heat shock proteins on all days post infection (Patterson and Kapoor, 1995).

^{35}S labeling of the proteins produced in the haemolymph of *S. gregaria* over the course of infection revealed interesting changes in protein production. The 32 kDa protein induced in *S. gregaria* haemolymph on day two of infection is likely to be a response by the locust to infection. This could be the same protein as the 33.4 kDa protein described by Gillespie (1995) which appeared in his experiments on the third day of infection. A number of proteins have been found to be induced in the haemolymph of insects in response to microbial invasion; lysozymes, bactericidal peptides, phenoloxidase, attacins, protease inhibitors and lectins (Kanost *et al.*, 1990). The 32kDa protein produced in *S. gregaria* haemolymph may be an anti-microbial, such as the serine protease inhibitors produced by *Bombyx mori* in response to infection with *Nomuraea rileyi*. These inhibitors are in the size range of 7 - 60kDa (Boucias and Pendland, 1987). Alternatively it could be a lysozyme like the one induced in *Spodoptera exigua* in response to injection with bacterial endotoxin. However this lysozyme was not found to be induced in response to *Beauvaria bassiana* infection and if it was induced by artificial means it was found to have no effect on the development of *Beauvaria bassiana* (Boucias *et al.*, 1994). Other antimicrobials are produced constitutively and would not be detected in response to infection, such as the anti-fungal protein produced by *Sarcophaga peregrina* (Iijima *et al.*, 1993). Gillespie (1995) proposed that a similar protein which he detected in the haemolymph of *S. gregaria* infected with *M. flavoviride* may be an α-glucosidase like the one detected in *Manduca sexta* mycosed with *M. anisopliae* (Cobb,1997).

In the mulberry silkworm heat shock at 41°C leads to the induction of a 93kDa protein in the haemolymph. It is interesting to note that on day 4 of infection with *M. flavoviride* a similar sized protein (94.4kDa) was manufactured in abundance (although this protein was also found in smaller quantities in the non-infected insects haemolymph). This is unlikely to be a heat shock protein (that is a molecular chaperone induced by heat) as these are usually intracellular and could be damaging to the host if released. However it may be that this protein is some other form of defense or stress management used by insects.

On day four of infection there is a radical change in the pattern of protein production in the haemolymph of *S. gregaria*. It is proposed that these proteins may be primarily produced by *M. flavoviride* as by this point in infection it the fungus has probably overcome the host's defense system and is growing prolifically in the haemolymph. If equal protein loading of haemolymph were used in each lane, the proteins produced in the other infected haemolymph samples could also be detected in the day 4 infected haemolymph (results not shown). This demonstrates that the overproduction of a few proteins in the haemolymph of infected insects on day 4 is masking the production of other proteins. On day 4 of infection the degree of incorporation of ^{35}S was also increased 10 fold. It is interesting that such a high protein production rate was found on day four of infection as previous work has shown that there is a steady drop in the amount of protein detected in infected locust haemolymph with time (Gillespie, 1995). Gillespie found a 17% reduction in the quantity of protein detected by day four of infection. This would suggest that the speed of protein turnover on the fourth day of infection must be substantially increased.

One problem of this method of studying protein production in response to infection is that proteins may additionally be induced as an injury response to the injection of ^{35}S methionine in non-infected insects. It has been shown in *Sarcophaga peregrina* that all proteins induced in response to micro-organisms can also be induced in response to

damage of the integument by a hypodermic needle (Kanost *et al.*, 1990). If this is also true in *S. gregaria* we may be getting a biased picture of normal protein production in the haemolymph, although comparing infected and non-infected haemolymph provides a control.

4.4.4 *hsp70* in *Metarhizium anisopliae* and *Metarhizium flavoviride*

All results so far indicate that *M. anisopliae* isolate ME1 has only one copy of the *hsp70* gene and from the PCR sequence it is evident that this gene contains an intron. It is extremely unusual for an organism to only have one copy of the *hsp70* gene, usually organisms possess a number of different copies of the *hsp70* gene which are regulated differently. For example, *Saccharomyces cerevisiae* has 8 different *hsp70* genes which are divided into four different groups depending on their function and location (Schilke *et al.*, 1996). In addition, introns are only usually found in those genes which are constitutive (the *hsc70*s) as there is often a block in the splicing of mRNA precursors at higher temperatures. However, there are now a number of exceptions to this theory. Indeed, further work carried out on the sequencing of the *M. anisopliae hsp70* gene by A. Bailey (pers. comm.) has shown the presence of HSEs (heat shock elements) which give very good evidence that the cloned gene is indeed heat shock induceble. There is only one other description of an organism containing a single heat shock inducible *hsp70* gene and this is in the water fungus, *Blastocladiella emersonii.* This gene also contains an intron (although not at the same position as the one in *M. anisopliae*) and the splicing of this intron has been found to be highly thermoresistant (Stefani and Gomes, 1995). They also found that this gene contained several heat shock element (HSE) inverted repeats giving evidence that this gene is heat shock inducible. Other regulatory elements found included binding sites for ATF, Sp1 and two metal responsive elements. Sequencing of the genomic clone of *M. anisopliae* hsp70 from the λ library will be necessary to further study the regulation of this *hsp70* gene. It is possible that in *M. anisopliae* and *B. emersonii* these single copy genes are responsible for both constitutive expression and heat shock induced expression. This situation also

gives rise to a problem in the classification of the gene and its resultant protein. Typically those proteins which are constitutively expressed are referred to as HSCs or heat shock cognates and those which are inducable are referred to as HSPs. In the case of *M. anisopliae* and *B. emersonii* we cannot make this distinction.

The evidence of differences in the number of *hsp70* genes in *M. flavoviride* and *M. anisopliae* is contradictory. From PCR and rtPCR it would appear that there are at least 2 extra genes in *M. flavoviride*, however the Southern blot revealed the presence of only one band in each lane. In order to clear up this discrepancy the Southern blot needs to be repeated and probed again at a lower stringency.

The reasons for the differences in induction of different RNA species in *M. flavoviride* under different stress conditions as revealed by RT-PCR are not known. It is obvious that different stresses induce the translation of different heat shock protein-like mRNAs in *M. flavoviride*. It is probable there are many different promotors on the *hsp70* gene which respond differently to each of the stresses.

However, the results from the RT-PCR are open to some suspicion because none of the products appear to contain any introns, despite their larger than average size for *hsp70* genes. It is possible that there was some DNA contamination in the reactions, although the method of preparation and different levels of expression under each condition make this unlikely. This experiment needs to be repeated again including control reactions without reverse transcriptase and the resulting DNA products blotted and probed.

One of the proposed mechanisms for a similar variation in induction HSP70 in murine cortical astrocytes is that it is due to differences in the amount of free HSP70. On the application of stress, HSP70 becomes bound to denatured proteins and prevents them from aggregating. Different stresses will affect the amount of denatured protein present (for example, in murine astrocytes heat stress causes more proteins to denature than nutrient limitation does) and hence lead to differences in the amount of free HSP70 (Bergeron *et al.*, 1996). As described in the introduction to this Chapter, the amount of

free HSP70 in the cell is thought to be one of the factors responsible for regulating the level of response. Therefore the less free HSP70 there is the greater the transcription of any gene which contains the HSE. The *hsp70* gene in *M. flavoviride* probably has many different regulatory elements which are affected differently by the type of damage caused by each stress.

5. General Discussion

In 1989 a collaborative research programme was set up to examine the potential of various biological control agents against locusts and grasshoppers. Since this time, *Metarhizium flavoviride* isolate IMI 330189 has been identified as showing the greatest promise as a BCA. A number of problems have been encountered during its development, many of which have now been overcome. For example, the use of oil formulation has provided a number of benefits, not least of which is the improved ability of the fungus to infect insects at lower humidities (Bateman *et al.*, 1993).

Environmental constraints are frequently documented as being one of the major limiting factors in the commercial development of entomopathogenic fungi and this is a particular problem when considering the control of locusts and grasshoppers as they inhabit some of the most arid and inhospitable regions of the world. This research set out to investigate some of the implications of high temperature on locust control.

Work in Chapter 2 showed that high temperature has an adverse effect on the ability of *M. flavoviride* to germinate and produce appressoria *in vivo*. This work used a maximum temperature at 35°C. However, in the field temperatures can rise even higher and may cause very severe problems. During a locust swarm in Madagascar, temperatures of up to 55°C were recorded, with the temperature being over 40°C for at least 8 hours a day (Bateman *et al.*, 1994). These high temperatures are certain to have a strong influence on the infectivity of *M. flavoviride*.

The effects of these high temperatures on the ability of *M. flavoviride* to cause infection were illustrated in Chapter 3, where the disease was shown to be completely inhibited at 40°C. It is likely that at a constant temperature of 40°C *M. flavoviride* would be unable to germinate and grow at all. Indeed this has been illustrated *in vitro* during this study.

However, temperatures as consistently high as this are unlikely to occur in the field. It is more likely that the temperatures will fluctuate. The problem caused by fluctuating

temperatures were not investigated during this study but will require investigation in the future. Milder temperatures overnight will allow the spores to germinate. As the temperature increases during the day they may have an even more devastating effect on the germlings as these are likely to be more sensitive to environmental temperature and humidity than conidia.

These temperature constraints are not usually seen in chemical insecticides, which will give rise to problems persuading farmers to switch to this sort of biological control system. Therefore it is necessary to attempt to improve the efficiency of *M. flavoviride* at these higher temperatures.

Although combined application of with a chemical insecticide is one possible solution to overcoming temperature constraints, it does defeat some of the purposes of using a BCA. As has been shown in this study, the affect of teflubenzuron on *Schistocerca gregaria* is greatly enhanced by high temperatures and this has been shown to be the case with a number of other insecticides (Ruigt, 1985).

Although teflubenzuron applied with *M. flavoviride* was not found to be synergistic at 25 and 30°C, it did show synergy at 35°C. As it is these higher temperatures that are a problem combined applications may have some benefits. However, complexities in the relationships between temperature, teflubenzuron, *M. flavoviride* and instar mean that use of teflubenzuron as part of an IPM scheme could not be recommended from the results in this study. However, initial experiments have shown that combined oral doses may be a possibility and should be further investigated.

Other possible solutions to the problems caused by excessively high temperatures were investigated in Chapters 2 and 4. Subculturing the fungi at a higher temperature did improve the ability of the conidia to germinate at higher temperatures. However, results were mixed and there was a loss in other attributes (such as a severely reduced sporulation and poor growth). Improving the ability of the fungus to infect at higher temperatures should not be done at the cost of other qualities. Other work has shown

that that although conidia of a plant pathogen produced at higher temperatures showed very rapid growth they had a very low infectivity (Abbas *et al.*, 1995). The same situation may occur with *M. flavoviride* and further investigations may show that the conidia able to germinate at higher temperatures have lost a considerable degree of virulence.

Another possibility is to alter the spraying times of the fungus to avoid excessive midday temperatures by spraying in the early morning or the evening.

The fact that the conidia were found to germinate fastest on the hindwings of adult locust also has implications for spraying technique. Work carried out by Seyoum (1994) showed that ULV spraying of flying adult locust with *M. flavoviride* in oil formulation resulted in the majority of the formulation landing on the wings of the locust (no differentiation was made between the fore and hind wings). These results would suggest that LD_{50}s and LT_{50}s may be minimized by spraying flying swarms so that most of the formulation land on the wings. This would have the additional benefit that any spores landing on the hindwing would be in a much more protected environment. However, it is not yet known what effect each body part has on infection and conidia applied to the wing may be very inefficient at causing disease.

Another possible solution to the problem of excessive temperature which was investigated was the induction of thermotolerance. Thermotolerance was induced in *M. flavoviride* spores in aqueous solution and was demonstrated to be due to the production of proteins (as the effects could be inhibited by the addition of cycloheximide). However, the same thermotolerance has not been induced in spores in oil. These spores have a very high resilience to temperature while in formulation but this is not maintained when they begin to germinate.

It may be possible to increase thermotolerance before this stage by altering the growth media or conditions under which spores are produced. Hallsworth and Magan (1995) demonstrated that *M. anisopliae* conidia grown on media supplemented trehalose had

increased intracellular trehalose levels. Other workers have shown that increased trehalose levels can have a drastic effect on improving the ability of fungi to survive high temperatures. Trehalose levels in *M. anisopliae* lead to a reduced germination rate and therefore this particular method is not suitable (Hallsworth and Magan, 1996). However, the induction of other chemicals and proteins which might protect against excessive temperatures is still a possibility.

From the results so far it would appear unlikely that HSP70 has a major role in protecting *M. flavoviride* at very high temperatures. However, the 106kDa protein that showed very high induction levels during heat shock may well be a member of the HSP100 family and may prove to be a more useful protein to use to increase thermotolerance. This HSP has previously been implicated in a number of fungi as being of prime importance in protecting the from high temperatures (Parsell *et al.*, 1994). HSP100 may therefore prove to be the best protein for further studies on improving thermotolerance in *M. flavoviride*.

However, HSP70 should not be discounted from further studies. It shows interesting variations between different isolates of *Metarhizium spp*. The *hsp70* gene in *M. anisopliae* is also extremely interesting as it has goes against many of the theories about the structure and function of *hsp70* genes. All the evidence so far points to it being a single copy gene. This is an extremely unusual condition and has only been described in *Blastocladiella emersonii* (Stefani and Gomes, 1995). The Western blots also suggest that this gene is heat shock inducible. Further evidence for heat shock inducibility has emerged as the genomic DNA has been sequenced and Heat Shock Elements (HSEs) have been found (unpublished).

Generally speaking, *hsp70* genes which are heat shock inducible do not contain introns. However, *M. anisopliaes hsp70* has been found to contain both an intron and HSEs. Finally, it appears that *M. anisopliaes hsp70* gene contains a number of extra base pairs not found in any other *hsp70* gene investigated (although they are contained in a

variable area of the gene). All these factors mean that although the *hsp70* may be of limited interest from the point of view of pathogenicity, it could prove extremely useful in furthering our knowledge of *hsp70* genes in general.

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Appendices

Appendix 1 - Culture Media

Culture media - *Metarhizium spp.*

Unless otherwise stated all media was autoclaved at 120 °C and 15 lbin^2 for 15 minutes.

¼ SDA (¼ strength Sabouraud Dextrose Agar)

D-glucose	10 gl^{-1}
Mycological peptone	2.5 gl^{-1}
Agar	20 gl^{-1}
Yeast extract	1.25 gl^{-1}

Add all ingredients and stir at 60 °C for 10 minutes. SDB is made as above, but the agar is omitted and it is not heated prior to autoclaving (Sabouraud, 1910).

Selective media

This was prepared as described for ¼ strength SDA except that 1ml of filter sterilised chloramphenicol (0.4$mgml^{-1}$) dissolved in ethanol and 1ml of filter sterilised cyclohexamide (0.5gl^{-1}) dissolved in water were added to 1l of media after autoclaving.

Modified Czapek Dox liquid medium

Czapek Dox liquid medium (Oxoid)	33.4 gl^{-1}
Casein hydrolysate (Oxoid)	2 gl^{-1}
Malt extract	2 gl^{-1}
Yeast extract	2 gl^{-1}
Mycological peptone	2 gl^{-1}

Make up to 1 l with distilled water, stir until dissolved.

Basal salts media

KH_2PO_4	1 gl^{-1}
$MgSO_4.7H_2O$	0.5 gl^{-1}
Trace element solution	10 ml
MES buffer	50 mM

Make up to 800 ml with distilled water, pH to 6.5 and make up to 1 l.

Trace elements solution

$ZnSO_4$	10 gl^{-1}
$NaMoO_4.2H_2O$	0.2 gl^{-1}
$FeSO_4$	2 gl^{-1}
$CuSO_4.5H_2O$	0.2 gl^{-1}
$MnCl.4H_2O$	0.2 gl^{-1}

Add sequentially and mix until dissolved.

Culture Media - *Escherichia coli*

LB media (Luria-Bertani medium)

Bacto-tryptone	10 gl-1
Bacto-yeast extract	5 gl-1
NaCl	5 gl-1

Dissolve in 950 ml of distilled water. Adjust to pH 7.0 with 5 M NaOH and adjust volume to 1 l. For plates add 15 g of agar.

2 × YT media

Bacto-tryptone	16 gl-1
Bacto-yeast extract	10 gl-1
NaCl	5 gl-1

Make up as described above for LB medium.

Transformation plates

Make up 1l of LB agar, sterilise. Cool to ~40°C and 100mg of ampicilin. Pour plates. When dry spread 100 µl of 50 mgml-1 Xgal and 100µl of 0.1M IPTG.

Appendix 2 - Locust feed

Trisulfa stock solution

sodium sulamtazine	4.25%
Sodium suldathiazole	3.65%
Sodium sulfamerazine	3.13%

Trisulfa stock was added to drinking water at 5% concentration and 70ml were sprinkled over 930g of bran.

Appendix 3 - DNA Reagents

Denaturing solution

NaOH	0.5 M
NaCl	1.5 M

Fungal DNA extraction buffer

Tris-HCl (pH 8.0)	200 mM
NaCl	250 mM
EDTA (pH 8.0)	25 mM
SDS	0.5 % w/v

GTE

Glucose	50 mM
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Autoclave and store at 4 C.

6 x loading buffer

Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Glycerol	3 ml
Water	7 ml

Neutralizing solution

Tris-HCl (pH 7.0)	1 M
NaCl	1.5 M

Southern blot pre-hybridization solution

20 % SDS	30 ml
1 M Na_2HPO_4	26 ml
Distilled water	44 ml
Skimmed milk	1 g

Mix together until milk powder dissolves.

20 × SSC

NaCl	175.3 gl^{-1}
Sodium citrate	88.2 gl^{-1}

Dissolve both constituents in 800 ml of distilled water. Adjust the pH to 7.0 with 10 N NaOH. Make up to 1 l.

5 x TBE

Tris	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml

TE

Tris-HCl (pH 7.4)	10 mM
EDTA (pH 8.0)	1 mM

Appendix 4 - PCR reagents

PCR

	Initial Concentration	Final Concentration	Volume used in reaction
ME1 DNA	50 ng	2 ng	2 µl
dNTPs	2 mM	200 µM	5 µl
$MgCl_2$	25 mM	1.5 mM	3 µl
buffer	10 ×	1 ×	5 µl
Taq	5 U	1 U	0.2 µl
Oligonucleotides	100 pmolµl^{-1}	2 pmolµl-1	1 µl each
Sterile de-ionised water			32.8 µl
Total			50 µl

Appendix 5 - λ library reagents

SM buffer

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g
1 M Tris-HCl (pH 7.5)	50 ml
2 % gelatin	5 ml

Make up to 1 l and autoclave.

Appendix 6 - RNA solutions

DEPC treated water

DEPC	1 ml
distilled water	1 l

Put both solutions into an RNase free beaker and leave overnight. Autoclave for 30 minutes at 15 lbin^2.

5 × formaldehyde running buffer

MOPS (pH7.0)	20.6 g
Sodium acetate	40 mM
5 mM EDTA (DEPC treated)	10 ml
DEPC treated water	1 l

Dissolve the sodium acetate in 800 ml of DEPC treated water. Add the MOPS and adjust the pH to 7.0 with 2 N NaOH. Add 10 ml of the EDTA before adjusting the final volume to 1 l.

50 x Denhardts solution

BSA	1 ml
Ficoll 400	1 ml
Polyvinyl pyrolidone 300	1 ml

Make up to 100 ml with sterile distilled water.

RNA hybridization buffer

formamide	25 ml
sodium pyrite	1.1 g
20 x SSC	12.5 ml
Denhardts solution	5 ml
20 % SDS	250 µl
ssDNA	1 ml
polyA	10 µl

Appendix 7 - Protein solutions

Protein extraction buffer

0.1 M PMSF	2 ml
Tris	0.48 g
NaCl	0.23 g

Add approx. 100 ml distilled water, pH to 7.5 with HCl and make volume up to 200 ml.

Sample loading buffer

Distilled water	4 ml
0.5 M Tris-HCl (pH 6.8)	1 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
B -mercaptoethanol	0.4 ml
0.05% bromophenol blue	0.2 ml

Stacking gel buffer stock

Tris-HCl (pH6.8)	6 g

Make up to 100 ml with distilled water.

Resolving gel buffer stock

Tris-HCl (pH 8.8)	36.3 g

Make up to 100 ml with distilled water.

10x Running buffer

Tris	30.3 g
Glycine	144g
SDS	10g

Make up to 1l with distilled water. Should have a pH of about 8.4

Coomassie blue stain

Methanol	500 ml
Distilled water	425 ml
Acetic acid	75 ml
Coomassie blue	5 g

Destain

Methanol	450 ml
Distilled water	460 ml
Acetic acid	90 ml

Tris-Glycine buffer (pH8.3)

Tris	3.03 g
Glycine	14.4 g

Make unto 1 l (do not add acid or base to adjust pH)

Towbin buffer

Tris-Glycine buffer (pH 8.3)	400 ml
Distilled water	400 ml
Methanol	200 ml

TBS (Tris -buffered saline)

Tris-HCl (pH7.5)	2.42 g
NaCl	29.2 g

Make unto 1 l with distilled water.

TTBS (Tris-buffered saline with Tween)

TBS	1 l
Tween-20	1 ml

Appendix 8 - GENSTAT programmes

LD_{50} values

```
UNITS {16}                                  Defines length of variate.
SCAL t;4                                    Defines the number of units for each variate.
FACT [levels=#t] trial                      Assigns qualitative variables for each trial.
READ trial, conc, total, kill               Inputs data in the format specified.
1 0 20 0                                    First number is a numerical identifier for each trial.
1 0.1 20 0
1 1.0 20 3
1 10 20 3
2 0 20 0
2 0.1 20 0
2 1.0 20 3
2 10 20 3
3 0 20 0
3 0.1 20 0
3 1.0 20 3
3 10 20 3
4 0 20 0
4 0.1 20 0
4 1.0 20 3
4 10 20 3
:
CALC [print=s] logconc=LOG10(conc)          Calculates the log 10 for each concentration.
PROBITAN [print=m,s,e,c,f]                  Analyses all trials as one. Uses probit analysis, a
kill;dose=logconc;nbin=total                form of non-linear regression analysis which
                                            assumes binomial distribution.
EXPR LD50; !e(Ldose50=10**'LD50')           Calculates the LD50 value.
RFUCTION [print=est,se;calc=LD50] Ldose50    Prints the results from the above calculations as
                                            defined.
PROBITAN
[print=m,s,e,c,f;group=trial]kill;dose=logconc;nbin=
total
FOR i=1...t                                 Start of loop,  continued until all trials analysed.
TEXT dumt
PRINT [channel=dumt; squash=yes;iprint=*]
'LD50[',i,']';field=4,1,0;dec=
skip=0
EXPR ld10[i];!e(Ldose50[i]=10**(#dumt))
RFUNCTION [print=est,se;calc=LD10] Ldose50[i]
ENDFOR                                      End of loop.
PROBITAN                                    Analyses each trial separately assuming different
[print=m,s,e,c,f;group=trial;sep=slope]kill;dose=logc slopes for each.
onc;nbin=total
FOR i=1...t
RFUCTION [print=est,se;calc=LD10[i]] Ldose50
ENDFOR
PROBITAN                                    Analyses each trial separately assuming all have
```

```
[mort=est;print=m,s,e,c,f;group=trial]kill;dose=logc
onc;nbin=total
FOR i=1...t
RFUCTION [print=est,se;calc=LD10[i]] Ldose50[i]
ENDFOR
FOR i=1...t
RFUCTION [print=est,se;calc=LD10[i]] Ldose50[i]
ENDFOR
STOP
```

the same slope.

LT_{50} values

```
vari[valu=1...6,100]day
point[nv=24]count,diffs

variate[nv=7]count[]
read[ser=y]count[]
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:
0 0 0 1 3 4 5:
0 0 0 0 3 4 5:

calc lday=log(day)

restcount[];day.lt.100
for i=1...24
        graph count[i];day
        graph count[i]; lday
endfor
rest count[]
calc diffs[]=diff(count[])

vari [valu=(#days)24]Time
```

Describes the units for each set of data
Defines the number of variables and adds extra columns for data to be calculated.
Describes the number of values for each variate.
inputs data

Calculates the log of the day.

Produces graphs of numbers dead against the time
Produdes graphs of numbers dead against log time

Calculates the change in counts between each column

```
fact[lev=day;valu=7(1...6)4]Rep          Catogarises data for replicates
fact[lev=6;valu=42(1...4)]Treat          Catogarises data for treatments
print Diffs, Time,Rep,Treat              Prints calculated values
model [dist=poisson;link=log]Diffs       Models number dead assuming Poison distribution
terms(Treat/Rep)*Time                    Terms used in regression model.
fit[print=*]Treat/Rep                    Fit regression line to model
add [print=*]Time                        Add time.
add[print=acc]Time*Treat
vari[nval=24]b,m
for i=1...24
        cumdistribution
count[i];time=day;init=!(1,4)
        rkeep est=est;fit=f
        calc f=cum(f)
        restrict f,day;day.lt.100
        graph f,count[i];day;meth=c,p
        calc (b,m)$[i]=est$[1,2]
        restrict f,day
endfor

pointer [nv=4]allCount
calc allCount[1]= count[1]+ count[2]+ count[3]+
count[4]+ count[5]+ count[6]
& allCount[2]= count[7]+ count[8]+ count[9]+
count[10]+ count[11]+ count[12]
& allCount[3]= count[13]+ count[14]+ count[15]+
count[16]+ count[17]+ count[18]
& allCount[4]= count[19]+ count[20]+ count[21]+
count[22]+ count[23]+ count[24]
for i=1...4
        cumdistribution
         allCount[i];time=day;init=!(1,4)
        rkeep est=est;fit=f
        calc f=cum(f)
        restrict f,day;day.lt.100
        graph f, allCount[i];day;meth=c,p
        restrict f,day
endfor                                   Fits data for each temperature and draws a graph

fact [lev=6;values=(1...6)4]rep
fact [lev=4;values=6(1...4)]treat
block rep
treat treat
anova [fprob=y]b,m
stop
```

Synergy

```
units [36]                               Length of variate.
open 'synergy'; ch=2                     Opens data from "synergy" folder.
read [ch=2]m                             Reads data into programme.
factor [lev=2]block                      Assigns categorical variables for each item, defined
                                         by columns of data.
factor [lev=3]temp
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```
factor [lev=3]tefl
factor [lev=2]spores
variate [values=36(5)]n
generate block,temp,tefl,spores          Generate a table of data based on temperature,
                                         teflubenzuron concentration and spore
                                         concentration.
tabulate [print=total;class=spores,tefl]m  Tabulates totals for each column and row.
Model [dist=binomial]m;nbinomial=n       Describes a model for the data based on binomial
                                         distribution.

terms tefl*spores*block
fit block                                Fits pattern to table.
add temperature                          Looks at the effects of temperature and any values
                                         that deviate from the pattern.
add tefl                                 Looks at the effect of teflubenzuron and for values
                                         that don't follow the pattern.
add tefl                                 Looks at the effect of teflubenzuron and for values
                                         that don't follow the pattern.
add [print=est,fitt,acc]spores*tefl      Looks for the interaction between spores and
                                         insecticide and any values that don't follow the
                                         expected.

stop
```